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(71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; Fruit Street, Boston, MA 02114 (US).

(72) Inventors: YUAN, Junying; 22 Fairlee Road, Newton, MA 02168 (US). MIURA, Masayuki; 11 Orchard Road, Brookline, MA 02146 (US).

(74) Agents: GOLDSTEIN, Jorge, A. et al.; Sterne, Kessler, Goldstein & Fox, Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

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(54) Title: PROGRAMMED CELL DEATH GENES AND PROTEINS

(57) Abstract

This invention relates to genes involved in regulating programmed cell death, the proteins encoded by such genes and methods for controlling programmed cell death by regulating the activity of the cell death gene products.

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PROGRAMMED CELL DEATH GENES AND PROTEINS

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Cross-Reference to Related Applications

This application is a continuation-in-part application of U.S. Application No. 08/080,580, filed June 24, 1993.

Field of the Invention

The invention is in the field of molecular biology as related to the control of programmed cell death.

Description of the Background Art

Cell death occurs as a normal aspect of animal development as well as in tissue homeostasis and aging (Glucksmann, A., *Biol. Rev. Cambridge Philos. Soc. 26*:59-86 (1950); Ellis *et al., Dev. 112*:591-603 (1991)). Naturally occurring cell death acts to regulate cell number, to facilitate morphogenesis, to remove harmful or otherwise abnormal cells and to eliminate cells that have already performed their function. Such regulated cell death is achieved through a cell-endogenous mechanism of suicide, termed programmed cell death or apoptosis (Wyllie, A. H., in *Cell Death in Biology and Pathology*, Bowen and Lockshin, eds., Chapman and Hall (1981), pp.

9-34). Programmed cell death or apoptosis occurs when a cell activates this internally encoded suicide program as a result of either internal or external signals. The morphological characteristics of apoptosis include plasma membrane blebbing, condensation of nucleoplasm and cytoplasm and degradation of chromosomal DNA at inter-nucleosomal intervals. (Wyllie, A. H., in *Cell Death in Biology and Pathology*, Bowen and Lockshin, eds., Chapman and Hall (1981), pp. 9-34). In many cases, gene expression appears to be required for programmed cell death, since death can be prevented by inhibitors of RNA or protein synthesis (Cohen *et al., J. Immunol. 32*:38-42 (1984); Stanisic *et al., Invest. Urol. 16*:19-22 (1978); Martin *et al., J. Cell Biol. 106*:829-844 (1988)).

The genetic control of programmed cell death has been well-elucidated by the work on programmed cell death in the nematode *C. elegans*. Programmed cell death is characteristic and widespread during *C. elegans* development. Of the 1090 somatic cells formed during the development of the hermaphrodite, 131 undergo programmed cell death. When observed with Nomarski microscopy, the morphological changes of these dying cells follow a characteristic sequence. (Sulston *et al., Dev. Biol. 82*:110-156 (1977); Sulston *et al., Dev. Biol. 100*:64-119 (1983)). Fourteen genes have been identified that function in different steps of the genetic pathway of programmed cell death in this nematode (Hedgecock *et al., Science 220*:1277-1280 (1983); Ellis *et al., Cell 44*:817-829 (1986); Ellis *et al., Dev. 112*:591-603 (1991); Ellis *et al., Genetics 112*:591-603 (1991b); Hengartner *et al., Nature 356*:494-499 (1992); Ellis *et al., Dev. 112*:591-603 (1991)). Two of these genes, *ced-3* and *ced-4*, play essential roles in either the initiation or execution of the cell death program. Recessive mutations in these genes prevent almost all of the cell deaths that normally occur during *C. elegans* development. Additional support for the view that *ced-3* and *ced-4* cause cell death comes from the genetic analysis of mosaics (Yuan *et al., Dev. Biol. 138*:33-41 (1990)). The *ced-4* gene encodes a novel protein that is expressed

primarily during embryogenesis, the period during which most programmed cell deaths occur (Yuan *et al., Dev. 116*:309-320 (1992)).

A gain-of-function mutation in *ced-9* prevents the normal programmed cell death, while mutations that inactivate *ced-9* are lethal, suggesting that *ced-9* may act by suppressing programmed cell death genes in cells that normally do not undergo programmed cell death (Hengartner, M., *et al., Nature 356*:494-499 (1992)). The *ced-9* gene encodes a protein product that shares sequence similarity with the mammalian proto-oncogene and cell death suppressor *bcl-2* (Hengartner, M., *et al., Cell 76*:665-676 (1994)). The lethality of *ced-9* loss-of-function mutations can be suppressed by mutations in *ced-3* and *ced-4*, indicating that *ced-9* acts by suppressing the activity of *ced-3* and *ced-4*. Genetic mosaic analyses indicate that *ced-3* and *ced-4* likely act in a cell-autonomous fashion within dying cells, suggesting that they might be cytotoxic proteins and/or control certain cytotoxic proteins in the process of programmed cell death (Yuan, J., *et al., Dev. Bio. 138*:33-41 (1990)). The 549 amino acid sequence of the *ced-4* protein, deduced from cDNA and genomic clones, contain two regions that are similar to the calcium-binding domain known as the EF-hand (Kretsinger, 1987); however, it is still not clear at present whether calcium plays a role in regulating *ced-4* or programmed cell death in *C. elegans*.

Summary of the Invention

In the present invention, the *ced-3* gene has been cloned and sequenced and the amino acid sequence of the protein encoded by this gene is disclosed. Structural analysis of the *ced-3* gene revealed that it is similar to the enzyme interleukin-1β converting enzyme ("*ICE*") and that overexpression of the murine interleukin-1β converting enzyme ("*mICE*") causes programmed cell death in vertebrate cells. Based upon these results, a novel method for controlling programmed cell death in vertebrates by regulating the activity of *ICE* is claimed.

The amino acid sequence of the *ced-3* protein was also found to be similar to another murine protein, *nedd-2*, which is detected during early embryonic brain development, a period when many cells die. The results suggest that *ced-3*, *mICE* and *nedd-2* are members of a gene family which function to cause programmed cell death.

A new cell death gene, *mICE2,* has been discovered which appears to be in the same family as *ced-3*, *mICE*, and *nedd-2*. *mICE2* is distinguished from other previously identified cell death genes in that it is preferentially expressed in the thymus and placental cells of vertebrates. Thus, the invention is also directed to a newly discovered gene, *mICE2*, which is preferentially expressed in thymus and placental cells and which encodes a protein causing programmed cell death.

A comparison of the nucleotide sequences of *ced-3*, *mICE*, human *ICE*, *nedd-2* and *mICE2* indicates that they are part of a gene family whose members all promote programmed cell death. The identification of this family facilitated the isolation of the newly discovered cell death gene *Ice-ced 3* homolog (*Ich-1*). *Ich-1* is homologous with the other cell death genes described above and particularly with *nedd2*. Based upon its structure and the presence of a QACRG sequence characteristic of the active center of cell death genes, *Ich-1* was identified as a new member of the *ced-3/ICE* family. Thus, the present invention is directed to both the *Ich-1* gene sequence and the *Ich-1* protein. Also encompassed are vectors expressing *Ich-1* and host cells transformed with such vectors. Alternative splicing results in two distinct *Ich-1* mRNA species. Thus, the invention also encompasses these species, proteins produced from them, vectors containing and expressing the genes, and the uses described herein.

The inventors have also identified a new member of the *ICE/ced-3* family, *Ice-4*. *Ice-4* has at least two alternative splicing products. A full length cDNA of one of them from a mouse thymus cDNA library has been identified. It encodes a protein of 418 amino acids that is 38% identical with

murine *ICE*, 42% identical with murine *Ice-2*, 25% with murine *Ich-1*, and 24% identical with *C. elegans ced-3*.

The invention is thus directed to genomic or cDNA nucleic acids having genetic sequences which encode *ced-3*, *mICE2*, *Ich-1*, and *Ice-4*. The invention also provides for vectors and expression vectors containing such genetic sequences, the host cells transformed with such vectors and expression vectors, the recombinant nucleic acid or proteins made in such host/vectors systems and the functional derivatives of these recombinant proteins. The use of the isolated genes or proteins for the purpose promoting cell death is also part of the invention.

The invention is also directed to methods for controlling the programmed death of vertebrate cells by regulating the activity of interleukin-1β converting enzyme, "*ICE*." Such regulation may take the form of inhibiting the enzyme's activity, e.g. through the use of specific antiproteases such as *crmA*, in order to prevent cell death. In this way, it may be possible to develop cell lines which remain viable in culture for an extended period of time or indefinitely. Certain cells can only be maintained in culture if they are grown in the presence of growth factors. By blocking cell death, it may be possible to make such cells growth factor independent. Alternatively, *ICE* activity may be increased in order to promote cell death. Such increased activity may be used in cancer cells to antagonize the effect of oncogenes such as *bcl-2*.

Brief Description of the Figures

Figure 1 and 1A: Genetic and Physical Maps of the ced-3 Region on Chromosome IV

Figure 1 shows the genetic map of *C. elegans* in the region near *ced-3* with the cosmid clones representing this region depicted below the map.

nP33, *nP34*, *nP35*, *nP36*, and *nP37* are restriction fragment length polymorphisms (RFLP) between Bristol and Bergerac wild type *C. elegans* strains. C43C9, W07H6 and C48D1 are three cosmid clones tested for rescue of the *ced* phenotype of *ced-3(n717)* animals. The ability of each cosmid clone to rescue *ced-3* mutants and the fraction of independently obtained transgenic lines that were rescued are indicated on the right of the figure (+, rescue; -, no rescue; see text for data). The results indicate that *ced-3* is contained in the cosmid C48D1.

Figure 1A is a restriction map of C48D1 subclones. C48D1 was digested with *Bam*HI and self-ligated to generate subclone C48D1-28. C48D1-43, pJ40 and pJ107 were generated by partial digesting C48D1-28 with *Bgl*II. pJ7.5 and pJ7.4 were generated by *Exo*III deletion of pJ107. These subclones were assayed for rescue of the *ced* phenotype of *ced-3(n717)* animals (+, rescue; -, no rescue, -/+, weak rescue). The numbers in parentheses indicate the fraction of independently obtained transgenic lines that were rescued. The smallest fragment that fully rescued the *ced-3* mutant phenotype was the 7.5 kb pJ7.5 subclone.

Figure 2, 2A(i)-2A(v), 2B and 2C:
Genomic Organization, Nucleotide Sequence, and Deduced Amino Acid Sequence of ced-3

Figure 2 shows the genomic sequence of the *ced-3* region, as obtained from plasmid pJ107. The deduced amino acid sequence of the *ced-3* protein is based on the DNA sequence of *ced-3* cDNA pJ87 and upon other experiments described in the text and in Experimental Procedures. The 5' end of pJ87 contains 25 bp of poly-A/T sequence (not shown), which is probably a cloning artifact since it is not present in the genomic sequence. The likely start site of translation is marked with an arrowhead. The SL1 splice acceptor site of the *ced-3* transcript is boxed. The positions of 12 *ced-3* mutations are indicated. Repetitive elements are indicated as arrows above the relevant

sequences. Numbers on the left indicate nucleotide positions, beginning with the start of pJ107. Numbers below the amino acid sequence indicate codon positions. Five types of imperfect repeats were found: repeat 1, also found in *fem-1* (Spence *et al., Cell 60*:981-990 (1990)) and *hlh-1* (Krause *et al., Cell 63*:907-919 (1990)); repeat 2, novel; repeat 3, also found in *lin-12* and *fem-1*; repeat 4, also found in *lin-12*; and repeat 5, novel. Numbers on the sides of the figure indicate nucleotide positions, beginning with the start of pJ107. Numbers under the amino acid sequence indicate codon positions.

Figure 2A(i) - Figure 2A(iv) contain comparisons of the repetitive elements in *ced-3* with the repetitive elements in the genes *ced-3, fem-1, hlh-1, lin-12, glp-1*, and the cosmids B0303 and ZK643 (see text for references). In the case of inverted repeats, each arm of a repeat ("for" or "rev" for "forward" or "reverse", respectively) was compared to both its partner and to individual arms of the other repeats. 2A(i): Repeat 1; 2A(ii): Repeat 2; 2A(iii): Repeat 3; 2A(iv): Repeat 4; and 2A(v): Repeat 5. The different *ced-3* sequences which appear in the comparisons are different repeats of the same repetitive element. The numbers "1a", "1b" etc. are different repeats of the same class of repetitive element.

Figure 2B shows the locations of the introns (lines) and exons (open boxes) of the *ced-3* gene as well as the positions of 12 *ced-3* mutations analyzed. The serine-rich region, the trans-spliced leader (SL1), the possible start of translation (ATG) and polyadenlyation (AAA) site are also indicated.

Figure 2C shows the cDNA sequence and deduced amino acid sequence of *ced-3* as obtained from plasmid pJ87.

Figure 3 and 3A:
Structure of the ced-3 Protein

Figure 3 shows a comparison of structural features of *ced-3* with those of the human interleukin-1β converting enzyme (*ICE*) gene. The predicted proteins corresponding to the *ICE* proenzyme and *ced-3* are represented. The

active site in *ICE* and the predicted active site in *ced-3* are indicated by the black rectangles. The four known cleavage sites in *ICE* flanking the processed *ICE* subunits (p24, which was detected in low quantities when *ICE* was purified (Thornberry *et al.*, 1992), p20, and p10) and two conserved presumptive cleavage sites in the *ced-3* protein are indicated with solid lines and linked with dotted lines. Five other potential cleavage sites in the *ced-3* protein are indicated with dashed lines. The positions of the aspartate (D) residues at potential cleavage sites are indicated below each diagram.

Figure 3A contains a comparison of the amino acid sequences of the *ced-3* proteins from *C. elegans*, *C. briggsae* and *C. vulgaris* and the human and mouse *ICE* and mouse nedd-2 proteins. Amino acids are numbered to the right of each protein. Dashes indicate gaps in the sequence made to allow optimal alignment. Residues that are conserved among more than half of the proteins are boxed. Missense *ced-3* mutations are indicated above the comparison blocks showing the residue in the mutant *ced-3* protein and the allele name. Asterisks indicate potential aspartate self-cleavage sites in the *ced-3* protein. Circles indicate known aspartate self-cleavage sites in human *ICE*. Residues indicated in boldface correspond to the highly conserved pentapeptide containing the active cysteine in *ICE*.

Figure 4:
Construction of Expression Cassettes of mICE-lacZ and ced-3-lacZ Fusion Genes

Figure 4 shows several expression cassettes used in studying the cellular effects of *ICE* and *ced-3* gene expression. The cassettes are as follows: pβactM10Z contains intact *mICE* fused to the *E. coli lacZ* gene (*mICE-lacZ*). pβactM11Z contains the P20 and P10 subunits of *mICE* fused to the *E. coli lacZ* gene (P20/P10-*lacZ*). pβactM19Z contains the P20 subunit of *mICE* fused to the *E. coli lacZ* gene (P20-*lacZ*). pβactM12Z contains the P10 subunit of *mICE* fused to the *E. coli lacZ* gene (P10-*lacZ*). pβactced38Z

contains the *C. elegans ced-3* gene fused to the *lacZ* gene (*ced-3-lacZ*). pJ485 and pβactced37Z contain a Gly to Ser mutation at the active domain pentapeptide "QACRG" in *mICE* and *ced-3* respectively. pβactM17Z contains a Cys to Gly mutation at the active domain pentapeptide "QACRG" in *mICE*. pactβgal' is a control plasmid (Maekawa *et al.*, *Oncogene* 6:627-632 (1991)). All plasmids use the β-actin promoter.

Figure 5:
Genetic Pathways of Programmed Cell Death in the Nematode C. elegans and in Vertebrates

In vertebrates, *bcl-2* blocks the activity of *ICE* thereby preventing programmed cell death. Enzymatically active *ICE* causes vertebrate cell death. In *C. elegans*, *ced-9* blocks the action of *ced-3/ced-4*. Active *ced-3* together with active *ced-4* causes cell death.

Figure 6:
mICE2 cDNA Sequence and Deduced Amino Acid Sequence

Figure 6 shows the nucleotide sequence of the *mICE2* cDNA sequence and the amino acid sequence deduced therefrom.

Figure 7 and 7A:
mICE2 Amino Acid Sequence

Figures 7 and 7A contain a comparison of the amino acid sequences of murine interleukin-1β converting enzyme (*mICE1*), human interleukin-1β converting enzyme (*hICE*), *mICE2* and *ced-3*.

Figure 8:
Ich-1 cDNA Sequence and Deduced Amino Acid Sequence

Figure 8 shows the nucleotide sequence of the *Ich-1* cDNA sequence and the amino acid sequence deduced therefrom.

Figure 9:
Potential QACRG Coding Region in the Mouse nedd2 cDNA

The reading frame proposed by Kumar *et al.* (*Biochem. & Biophys. Res. Comm. 185*:1155-1161 (1992)) is b. In reading frame a, a potential QACRG coding region is underlined.

Figure 10-10C:
Comparison of Mouse nedd2 and Ich-1 cDNA Sequences

Figure 10-10C contains a comparison of the mouse *nedd2* cDNA sequence (top strand) and the *Ich-1* cDNA sequence (bottom strand). The coding region for *nedd2* starts at basepair 1177.

Figure 11 and 11A:
Comparison of the Amino Acid Sequences of ced-3, ICE and Ich-1

Figure 11 contains a comparison of the amino acid sequences of *ced-3* and *Ich-1*. There is a 52% similarity between the sequences and a 28% identity.

Figure 11A contains a comparison of the amino acid sequences of *ICE* and *Ich-1*. There is a 52% similarity between the sequences and a 27% identity.

Figure 12A:
The cDNA Sequence of Ich-1_L and the Deduced Amino Acid Sequence of Ich-1_L Protein Product

The putative active domain is underlined.

Figure 12B:
The cDNA Sequence of Ich-1_S and the Deduced Amino Acid Sequence of Ich-1_S Protein Product

The intron sequence is underlined.

Figure 13:
The Schematic Diagram of Ich-1_L and Ich-1_S

Figure 14:

A comparison of the *Ich-1* protein sequence with the mouse *nedd*-2 protein, the human interleukin-1 β-converting enzyme (*ICE*) protein and *C. elegans ced-3* protein. Amino acids are numbered to the right of each sequence. Any residues in *nedd*-2, *ice* and *ced-3* that are identical with *Ich-1* protein are highlighted.

Figure 15:
Stable Expression of Ich-1_S Prevents Rat-1 Cells Induced by Serum Removal

Stable transfectants of Rat-1 cells expressing *bcl-2*, *crmA* or *Ich-1_S* were prepared as described in Experimental Procedures. Independent clones of both *Ich-1_S* positive and *Ich-1_S* negative were used. At time 0, exponentially growing cells were washed with serum-DMEM and dead cells were counted over time by trypan blue staining.

Figure 16:
The cDNA Sequence and Putative Ice-4 Protein Sequence

The putative first Met is marked with a dot.

Figure 17:
Comparison of Amino Acid Sequences of Ice-4 with ICE, Ice-2, Ich-1 and ced-3

Definitions

In the description that follows, a number of terms used in recombinant DNA (rDNA) technology or in the research area of programmed cell death are extensively utilized. In order to provide a clear and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Gene. A DNA sequence containing a template for a RNA polymerase. The RNA transcribed from a gene may or may not code for a protein. RNA that codes for a protein is termed messenger RNA (mRNA).

A "complementary DNA" or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

Cloning vector. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle, and into which DNA may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Markers, for example, are tetracycline

resistance or ampicillin resistance. The term "cloning vehicle" is sometimes used for "cloning vector."

Expression vector. A vector similar to a cloning vector but which is capable of expressing a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host and may additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

Programmed cell death. The process in which cell death is genetically programmed. Programmed cell death allows organisms to get rid of cells that have served a developmental purpose but which are no longer beneficial.

Functional Derivative. A "functional derivative" of *mICE2*, *Ich-1* (*Ich-1*$_L$ and *Ich-1*$_S$), or *Ice-4* is a protein which possesses a biological activity that is substantially similar to the biological activity of the non-recombinant. A functional derivative of may or may not contain post-translational modifications such as covalently linked carbohydrate, depending on the necessity of such modifications for the performance of a specific function. The term "functional derivative" is intended to include the "fragments," "variants," "analogues," or "chemical derivatives" of a molecule.

Fragment. A "fragment" is meant to refer to any variant of the molecule, such as the peptide core, or a variant of the peptide core.

Detailed Description of the Preferred Embodiments

Description

The present invention relates, *inter alia*, to isolated DNA encoding the *ced-3* protein of *C. elegans*, *mICE2*, *Ich-1*, and *Ice-4*. The invention also encompasses nucleic acids having the cDNA sequence of *ced-3*, *mICE-2*, *Ich-1*, and *Ice-4*. The invention also encompasses related sequences in other species that can be isolated without undue experimentation. It will be appreciated that trivial variations in the claimed sequences and fragments derived from the full-length genomic and cDNA genes are encompassed by the invention as well. The invention also encompasses protein sequences from *ced-3*, *Ich-1*, and *Ice-4*. It should also be understood that by *Ich-1* is intended both $Ich\text{-}1_S$ *and* $Ich\text{-}1_L$.

ced-3

The genomic sequence of the claimed gene encoding ced-3 is shown in Figure 2. The gene is 7,656 base pairs in length and contains seven introns ranging in size from 54 base pairs to 1,195 base pairs. The four largest introns as well as sequences 5′ to the START codon contain repetitive elements, some of which have been previously characterized in the non-coding regions of other *C. elegans* genes such as fem-1 (Spence *et al.*, *Cell 60*:981-990 (1990)) and hlh-I (Krause *et al.*, *Cell 63*:907-919 (1990)). A comparison of the repetitive elements in *ced-3* with previously characterized repetitive elements is shown in figures 2A(i) - 2A(v). The START codon of the *ced-3* protein is the methionine at position 2232 of the genomic sequence shown in Figure 2.

The cDNA sequence of *ced-3* shown in Figure 2C. The cDNA is 2,482 base pairs in length with an open reading frame encoding 503 amino

acids and 953 base pairs of 3' untranslated sequence. The last 380 base pairs of the 3' sequence are not essential for the expression of the *ced-3* protein.

In addition to encompassing the genomic and cDNA sequences of *ced-3* from *C. elegans*, the present invention also encompasses related sequences in other nematode species which can be isolated without undue experimentation. For example, the inventors have shown that *ced-3* genes from *C. briggsae* and *C. vulgaris* may be isolated using the *ced-3* cDNA from *C. elegans* as a probe (see Example 1).

The invention also encompasses protein products from the *ced-3* gene, gene variants, derivatives, and related sequences. As deduced from the DNA sequence, the *ced-3* protein is 503 amino acids in length and contains a serine-rich middle region of about 100 amino acids. The amino acid sequence comprising the claimed *ced-3* protein is shown in Figure 2 and Figure 2C. A comparison of the *ced-3* protein of *C. elegans* with the inferred *ced-3* protein sequences from the related nematode species *C. briggsae* and *C. vulgaris* indicates that the non-serine-rich region is highly conserved and that the serine-rich region is more variable. The non-serine-rich portion of the *ced-3* protein is also homologous with interleukin-1β converting enzyme (*ICE*), a cysteine protease that can cleave the inactive 31 kD precursor of IL-1β to generate the active cytokine (Cerretti *et al.*, *Science* 256:97-100 (1992); Thornberry *et al.*, *Nature* 356:768-774 (1992)). The C-terminal portions of both the *ced-3* and *ICE* proteins are similar to the mouse *nedd-2* protein, which is encoded by an mRNA expressed during mouse embryonic brain development and down-regulated in the adult brain (Kumar *et al.*, *Biochem. & Biophys. Res. Comm.* 185:1155-1161 (1992)). The results suggest that *ced-3* acts as a cysteine protease in controlling the onset of programmed cell death in *C. elegans* and that members of the *ced-3/ICE/nedd-2* gene family function in programmed cell death in a wide variety of species.

mICE-2

The cDNA sequence and deduced amino acid sequence of *mICE2* are shown in Figure 6. As expected, *mICE2* shows homology to both human and murine *ICE* as well as to *C. elegans ced-3* (see Figure 7 and 7A). In contrast to other cell-death genes that have been identified, *mICE2* is preferentially expressed in the thymus and placenta. Example 3 describes how the gene was obtained by screening a mouse thymus cDNA library with a DNA probe derived from human *ICE* under conditions of low stringency. Given the amino acid sequence and cDNA sequence shown in Figure 6, preferred methods of obtaining the *mICE2* gene (either genomic or cDNA) are described below.

Ich-1

nedd2, *ICE*, *mICE2* and *ced-3* are all members of the same gene family. This suggested that new genes might be isolated based upon their homology to identified family members.

nedd2 is a mouse gene which is preferentially expressed during early embryonic brain development (Kumar *et al., Biochem. Biophys. Res. Commun. 185*:1155-1161 (1992)). Since many neurons die during early embryonic brain development, it is possible that *nedd*-2 is a cell death gene.

Ich-1 is 2492 base pairs in length and contains an open reading frame of 441 amino acids (Figure 8). The C-terminal 130 amino acids of *Ich-1* are over 87% identical to mouse *nedd2*. However, *Ich-1* contains a much longer open reading frame and has the pentapeptide QACRG which is the active center of the proteins of the *ced-3/ICE* family. The results indicate that the cDNA isolated by Kumar may not have been synthesized from a fully processed mRNA and that the 5' 1147 base pairs which Kumar reported for *nedd2* cDNA may actually represent the sequence of an intron. The sequence reported by Kumar contains a region which could potentially code for QACRG

but these amino acids are encoded in a different reading frame than that indicated by Kumar (Figure 9). This suggests that Kumar made an error in sequencing.

The coding regions of *nedd2* and n37 are highly homologous (Figure 10). The amino acid sequence of the deduced n37 protein shares 28% identity with *ced-3* and 27% identity with *ICE* (Figure 11). The n37 protein was named *Ich-1*.

Ich-1 mRNA is alternatively spliced into two different forms. One mRNA species encodes a protein product of 435 amino acids, designated *Ich-1*$_L$, which contains amino acid sequence homologous to both P20 and P10 subunits of *ICE* as well as entire *ced-3* protein. The other mRNA encodes a 312 amino-acid truncated version of *Ich-1* protein, named *Ich-1*$_S$, that terminates 21 amino acid residues after the QACRG active domain of *Ich-1*. Expression of *Ich-1*$_L$ and *Ich-1*$_S$ has opposite effects on cell death. Overexpression of *Ich-1*$_L$ induces Rat-1 fibroblast cells to die in culture, while overexpression of the *Ich-1*$_S$ suppresses Rat-1 cell death induced by serum deprivation. Results herein suggest that *Ich-1* may play an important role in both positive and negative regulation of programmed cell death in vertebrate animals.

Ice-4

Ice-4 was identified based on its sequence homology with *ICE* and other isolated *ICE* homologs. Since the *Ice-4* clone isolated by PCR only contains the coding region for the C-terminal half of the *Ice-4* protein, a mouse thymus cDNA library was screened using the *Ice-4* insert. Among 2 million clones screened, 9 positive clones were isolated. The sequence herein is from one clone that contains the complete coding region for *Ice-4* gene.

Methods of Making

ced-3

There are many standard procedures for cloning genes which are well-known in the art and which can be used to obtain the *ced-3* gene (see e.g., Sambrook *et al.*, *Molecular Cloning, a Laboratory Manual*, 2nd edition, vol. 1-3, Cold Spring Harbor Laboratory Press, 1989). In Example 1, a detailed description is provided of two preferred procedures. The first preferred procedure does not require the availability of *ced-3* gene sequence information and is based upon a method described by Ruvkun *et al.* (*Molecular Genetics of Caenorhabditis Elegans Heterochromic Gene lin-14 121*: 501-516 (1988)). In brief, Bristol and Bergerac strains of nematode are crossed and restriction fragment length polymorphism mapping is performed on the DNA of the resulting inbred strain. Restriction fragments closely linked to *ced-3* are identified and then used as probes to screen cosmid libraries for cosmids carrying all or part of the *ced-3* gene. Positive cosmids are injected into a nematode strain in which *ced-3* has been mutated. Cosmids carrying active *ced-3* genes are identified by their ability to rescue the *ced-3* mutant phenotype

A second method for cloning *ced-3* genes relies upon the sequence information which has been disclosed herein. Specifically, DNA probes are constructed based upon the sequence of the *ced-3* gene of *C. elegans*. These probes are labelled and used to screen DNA libraries from nematodes or other species. Procedures for carrying out such cloning and screening are described more fully below in connection with the cloning and expression of *mICE2*, *Ich-1*, and *Ice-4*, and are well-known in the art (see, e.g., Sambrook *et al.*, *Molecular Cloning, a Laboratory Manual*, 2nd edition (1988)). When hybridizations are carried out under conditions of high stringency, genes are identified which contain sequences corresponding exactly to that of the probe. In this way, the exact same sequence as described by the inventors herein may

be obtained. Alternatively, hybridizations may be carried out under conditions of low stringency in order to identify genes in other species which are homologous to *ced-3* but which contain structural variations (see Example 1 for a description of how such hybridizations may be used to obtain the *ced-3* genes from *C. briggsae* and *C. vulgaris*).

The results in Example 2 demonstrate that the products of cell-death genes may be tolerated by cells provided they are expressed at low levels. Therefore, the *ced-3* protein may be obtained by incorporating the *ced-3* cDNA described above into any of a number of expression vectors well-known in the art and transferring these vectors into appropriate hosts (see Sambrook *et al.*, *Molecular Cloning, a Laboratory Manual*, vol. 3 (1988)). As described below in connection with the expression of *mICE2*, *Ich-1*, and *Ice-4*, expression systems may be utilized in which cells are grown under conditions in which a recombinant gene is not expressed and, after cells reach a desired density, expression may be induced. In this way, the tendency of cells which express *ced-3* to die may be circumvented.

mICE2, Ich-1, and Ice-4

DNA encoding *mICE2*, *Ich-1*, and *Ice-4* may be obtained from either genomic DNA or from cDNA. Genomic DNA may include naturally occurring introns. Moreover, such genomic DNA may be obtained in association with the 5′ promoter region of the sequences and/or with the 3′ transcriptional termination region. Further, such genomic DNA may be obtained in association with the genetic sequences which encode the 5′ non-translated region of the *mICE2*, *Ich-1*, and *Ice-4* mRNA and/or with the genetic sequences which encode the 3′ non-translated region. To the extent that a host cell can recognize the transcriptional and/or translational regulatory signals associated with the expression of the mRNA and protein, then the 5′ and/or 3′ non-transcribed regions of the native gene, and/or, the 5′ and/or 3′

non-translated regions of the mRNA, may be retained and employed for transcriptional and translational regulation.

Genomic DNA can be extracted and purified from any cell containing mouse chromosomes by means well known in the art (for example, see *Guide to Molecular Cloning Techniques*, S.L. Berger *et al.*, eds., Academic Press (1987)). Alternatively, mRNA can be isolated from any cell which expresses the genes, and used to produce cDNA by means well known in the art (*Id.*). The preferred sources for *mICE2* are thymus or placental cells. The mRNA coding for any of the proteins (i.e., *mICE2*, *Ich-1*, or *Ice-4*) may be enriched by techniques commonly used to enrich mRNA preparations for specific sequences, such as sucrose gradient centrifugation, or both.

For cloning into a vector, DNA prepared as described above (either human genomic DNA or preferably cDNA) is randomly sheared or enzymatically cleaved, and ligated into appropriate vectors to form a recombinant gene library. A DNA sequence encoding the protein or its functional derivatives may be inserted into a DNA vector in accordance with conventional techniques. Techniques for such manipulations are disclosed by Sambrook, *et al.*, *supra*, and are well known in the art.

In a preferred method, oligonucleotide probes specific for the gene are designed from the cDNA sequences shown in the Figures 6, 8, 12A, 12B, and 16. The oligonucleotide may be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and employed as a probe to identify and isolate the cloned gene by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Maniatis, T., *et al.* (In: *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982)), and by Hames, B.D., *et al.* (In: *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985)). Those members of the above-described gene library which are found to be capable of such hybridization are then analyzed to determine the extent and nature of the coding sequences which they contain.

To facilitate the detection of the desired coding sequence, the above-described DNA probe is labeled with a detectable group. This group can be any material having a detectable physical or chemical property. Such materials are well-known in the field of nucleic acid hybridization and any label useful in such methods can be applied to the present invention. Particularly useful are radioactive labels, such as ^{32}P, ^{3}H, ^{14}C, ^{35}S, ^{125}I, or the like. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. The oligonucleotide may be radioactively labeled, for example, by "nick-translation" by well-known means, as described in, for example, Rigby, P.J.W., *et al.*, *J. Mol. Biol. 113*:237 (1977) or by T4 DNA polymerase replacement synthesis as described in, for example, Deen, K.C., *et al.*, *Anal. Biochem. 135*:456 (1983).

Alternatively, oligonucleotide probes may be labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group. See, for example, Leary, J.J., *et al.*, *Proc. Natl. Acad. Sci. USA 80*:4045 (1983); Renz, M., *et al.*, *Nucl. Acids Res. 12*:3435 (1984); and Renz, M., *EMBO J. 6*:817 (1983).

For *Ich-1*, the isolation shown in the Examples was as follows. Two primers were used in the polymerase chain reaction to amplify *nedd2* cDNA from embryonic day 15 mouse brain cDNA (Sambrook et al., *Molecular Cloning, a Laboratory Manual*, vol. 3 (1988)). One primer had the sequence: ATGCTAACTGTCCAAGTCTA and the other primer had the sequence: TCCAACAGCAGGAATAGCA. The cDNA thus amplified was cloned using standard methodology. The cloned mouse *nedd2* cDNA was used as a probe to screen a human fetal brain cDNA library purchased from Stratagene. Such methods of screening and isolating clones are well known in the art (Maniatis, T., *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1982)); Hames, B.D., *et al.*, *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985)). A human *nedd*-2 cDNA clone was isolated that encodes a protein much longer than the mouse *nedd*-2 and contains amino acid sequences

homologous to the entire *ICE* and *ced-3* proteins. The isolated clone was given the name *Ice-ced 3* homolog or *Ich-1*.

The *Ich-1* cDNA may be obtained using the nucleic acid sequence information given in Figures 8, 12A, or 12B. DNA probes constructed from this sequence can be labeled and used to screen human gene libraries as described herein. Also as discussed herein, *Ich-1* may be cloned into expression vectors and expressed in systems in which host cells are grown under conditions in which recombinant genes are not expressed and, after cells reach a desired density, expression is induced. In this way, a tendency of cells which express *Ich-1* to die may be circumvented.

One method of making *Ice-4* is as follows. mRNA was isolated from embryonic day 14 mouse embryos using Invitrogens' microfast track mRNA isolation kit. The isolated mRNA was reverse transcribed to generate template for PCR amplification. The degenerate PCR primers were: *cIceB* {TG(ATCG)CC(ATCG)GGGAA(ATCG)AGGTAGAA} and *cTceAs* {ATCAT(ATC)ATCCAGGC(ATCG)TGCAG(AG)GG}. The PCR cycles were set up as follows: 1. 94°C, 3 min; 2. 94°C, 1 min; 3. 48°C, 2 min; 4. 72°C, 3 min; 5. return to "2" 4 cycles; 6. 94°C, 1 min; 7. 55°C, 2 min; 8. 72°C, 3 min; 9. return to "6" 34 cycles; 10. 72°C, 10 min; 11. end. Such PCR generated a band about 400bp, the predicted size of *ICE* homologs. The PCR products were cloned into T-tailed blunt-ended pBSKII plasmid vector (Stratagene). Plasmids that contain an insert were analyzed by DNA sequencing.

The *Ice-4* cDNA may also be obtained using the nucleic acid sequence information given in Figure 16. DNA probes constructed from this sequence can be labeled and used to screen human gene libraries as described herein. Also as discussed herein, *Ice-4* may be cloned into expression vectors and expressed in systems in which host cells are grown under conditions in which recombinant genes are not expressed and, after cells reach a desired density, expression is induced.

The methods discussed herein are capable of identifying genetic sequences which encode *mICE2*, *Ich-1*, and *Ice-4*. In order to further characterize such genetic sequences, and, in order to produce the recombinant protein, it is desirable to express the proteins which these sequences encode.

To express any of the genes herein (*mICE2*, *Ich-1*, *Ice-4*, and derivatives), transcriptional and translational signals recognizable by an appropriate host are necessary. The cloned coding sequences, obtained through the methods described herein, may be operably linked to sequences controlling transcriptional expression in an expression vector and introduced into a host cell, either prokaryote or eukaryote, to produce recombinant protein or a functional derivative thereof. Depending upon which strand of the sequence is operably linked to the sequences controlling transcriptional expression, it is also possible to express antisense RNA or a functional derivative thereof.

Expression of the protein in different hosts may result in different post-translational modifications which may alter the properties of the protein. Preferably, the present invention encompasses the expression of *mICE2*, *Ich-1*, and *Ice-4* or a functional derivative thereof, in eukaryotic cells, and especially mammalian, insect and yeast cells. Especially preferred eukaryotic hosts are mammalian cells either *in vivo*, or in tissue culture. Mammalian cells provide post-translational modifications which should be similar or identical to those found in the native protein. Preferred mammalian host cells include rat-1 fibroblasts, mouse bone marrow derived mast cells, mouse mast cells immortalized with Kirsten sarcoma virus, or normal mouse mast cells that have been co-cultured with mouse fibroblasts. Razin *et al.*, *J. of Immun. 132*:1479 (1984); Levi-Schaffer *et al.*, *Proc. Natl. Acad. Sci. (USA) 83*:6485 (1986) and Reynolds *et al.*, "Immortalization of Murine Connective Tissue-type Mast Cells at Multiple Stages of Their Differentiation by Coculture of Splenocytes with Fibroblasts that Produce Kirsten Sarcoma Virus," *J. Biol. Chem. 263*:12783-12791 (1988).

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains expression control sequences which contain transcriptional regulatory information and such sequences are "operably linked" to the nucleotide sequence which encodes the polypeptide.

An operable linkage is a linkage in which a coding sequence is connected to a regulatory sequence (or sequences) in such a way as to place expression of the coding sequence under the influence or control of the regulatory sequence. Two DNA sequences (e.g. the coding sequence of protein and a promoter) are said to be operably linked if induction of promoter function results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation; (2) interfere with the ability of regulatory sequences to direct the expression of the coding sequence, antisense RNA, or protein; or (3) interfere with the ability of the coding sequence template to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

The precise nature of the regulatory regions needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5′ non-transcribing and 5′ non-translating (non-coding) sequences involved with initiation of transcription and translation respectively, such as the TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5′ non-transcribing control sequences will include a region which contains a promoter for transcriptional control of the operably linked gene.

Expression of proteins of the invention in eukaryotic hosts requires the use of regulatory regions functional in such hosts, and preferably eukaryotic regulatory systems. A wide variety of transcriptional and translational regulatory sequences can be employed, depending upon the nature of the eukaryotic host. The transcriptional and translational regulatory signals can also be derived from the genomic sequences of viruses which infect eukaryotic cells, such as adenovirus, bovine papilloma virus, Simian virus, herpes virus,

or the like. Preferably, these regulatory signals are associated with a particular gene which is capable of a high level of expression in the host cell.

In eukaryotes, where transcription is not linked to translation, control regions may or may not provide an initiator methionine (AUG) codon, depending on whether the cloned sequence contains such a methionine. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis in the host cell. Promoters from heterologous mammalian genes which encode mRNA capable of translation are preferred, and especially, strong promoters such as the promoter for actin, collagen, myosin, etc., can be employed provided they also function as promoters in the host cell. Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, D., *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist, C., *et al.*, *Nature (London) 290*:304-310 (1981)); in yeast, the yeast *gal4* gene promoter (Johnston, S.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, P.A., *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)) or a glycolytic gene promoter may be used.

It is known that translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the proteins of the invention or functional derivatives thereof, does not contain any intervening codons which are capable of encoding a methionine. The presence of such codons results either in the formation of a fusion protein or a frame-shift mutation.

If desired, a fusion product of the proteins may be constructed. For example, the sequence coding for the proteins may be linked to a signal sequence which will allow secretion of the protein from, or the compartmentalization of the protein in, a particular host. Such signal sequences may be designed with or without specific protease sites such that the

signal peptide sequence is amenable to subsequent removal. Alternatively, the native signal sequence for this protein may be used.

Transcriptional initiation regulatory signals can be selected which allow for repression or activation, so that expression of operably linked genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical regulation, e.g., metabolite.

If desired, the non-transcribed and/or non-translated regions 3′ to the sequence coding for the proteins can be obtained by the above-described cloning methods. The 3′-non-transcribed region may be retained for transcriptional termination regulatory sequence elements; the 3′-non-translated region may be retained for translational termination regulatory sequence elements, or for those elements which direct polyadenylation in eukaryotic cells. Where native expression control signals do not function satisfactorily in a host cell, functional sequences may be substituted.

The vectors of the invention may further comprise other operably linked regulatory elements such as enhancer sequences, or DNA elements which confer tissue or cell-type specific expression on an operably linked gene.

To transform a mammalian cell with the DNA constructs of the invention many vector systems are available, depending upon whether it is desired to insert the DNA construct into the host cell chromosomal DNA, or to allow it to exist in extrachromosomal form. If the protein encoding sequence and an operably linked promoter are introduced into a recipient eukaryotic cell as a non-replicating DNA (or RNA) molecule, the expression of the protein may occur through the transient expression of the introduced sequence.

In a preferred embodiment, genetically stable transformants may be constructed with vector systems, or transformation systems, whereby *mICE2*, *Ich-1*, or *Ice-4* DNA is integrated into the host chromosome. Such integration may occur *de novo* within the cell or, in a most preferred embodiment,

through the aid of a cotransformed vector which functionally inserts itself into the host chromosome, for example, retroviral vectors, transposons or other DNA elements which promote integration of DNA sequences in chromosomes.

Cells which have stably integrated the introduced DNA into their chromosomes are selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector in the chromosome, for example the marker may provide biocide resistance, e.g., resistance to antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

In another embodiment, the introduced sequence is incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred eukaryotic plasmids include those derived from the bovine papilloma virus, vaccinia virus, SV40, and, in yeast, plasmids containing the 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., *et al.*, *Miami Wntr. Symp. 19*:265-274 (1982); Broach, J.R., In: *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, J.R., *Cell 28*:203-204 (1982); Bollon, D.P., *et al.*, *J. Clin. Hematol. Oncol. 10*:39-48 (1980); Maniatis, T., In: *Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Expression,* Academic Press, NY, pp. 563-608 (1980)), and are commercially available.

Once the vector or DNA sequence containing the construct(s) is prepared for expression, the DNA construct(s) is introduced into an

appropriate host cell by any of a variety of suitable means, including transfection. After the introduction of the vector, recipient cells are grown in a medium which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the protein, or in the production of a fragment of this protein. This expression can take place in a continuous manner in the transformed cells, or in a controlled manner, for example, expression which follows induction of differentiation of the transformed cells (for example, by administration of bromodeoxyuracil to neuroblastoma cells or the like). The latter is preferred for the expression of the proteins of the invention. By growing cells under conditions in which the proteins are not expressed, cell death may be avoided. When a high cell density is reached, expression of the proteins may be induced and the recombinant protein harvested immediately before death occurs.

The expressed protein is isolated and purified in accordance with conventional procedures, such as extraction, precipitation, gel filtration chromatography, affinity chromatography, electrophoresis, or the like.

The *mICE2*, *Ich-1*, and *Ice-4* sequences, obtained through the methods above, will provide sequences which not only encode these proteins but which also encode antisense RNA directed against *mICE2*, *Ich-1*, and *Ice-4*; the antisense DNA sequence will be that sequence found on the opposite strand of the strand transcribing the mRNA. The antisense DNA strand may also be operably linked to a promoter in an expression vector such that transformation with this vector results in a host capable of expression of the antisense RNA in the transformed cell. Antisense DNA and RNA may be used to interact with endogenous *mICE2*, *Ich-1*, or *Ice-4* DNA or RNA in a manner which inhibits or represses transcription or translation of the genes in a highly specific manner. Use of antisense nucleic acid to block gene expression is discussed in Lichtenstein, C., *Nature 333*:801-802 (1988).

Methods of Using

ced-3

The *ced-3* gene (as well as *ced-3* homologs and other members of the *ced-3* gene family) may be used for a number of distinct purposes. First, portions of the gene may be used as a probe for identifying genes homologous to *ced-3* in other strains of nematode (see Example 1) as well as in other species (see Examples 2 and 3). Such probes may also be used to determine whether the *ced-3* gene or homologs of *ced-3* are being expressed in cells.

The cell death genes will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. Among diseases and conditions which could potentially be treated are neural and muscular degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging. The discovery that *ced-3* is related to *ICE* suggests that cell death genes may play an important role in inflammation (IL-1β is known to be involved in the inflammatory response). Thus therapeutics based upon *ced-3* and related cell death genes may also be developed.

mICE2, Ich-1, and Ice-4

mICE2, *Ich-1*, and *Ice-4* will have the same uses as those described in connection with *ced-3* (above) and *ICE* (see below). The gene sequences may be used to construct antisense DNA and RNA oligonucleotides, which, in turn, may be used to prevent programmed cell death in thymus or placental cells. Techniques for inhibiting the expression of genes using antisense DNA or RNA are well-known in the art (Lichtenstein, C., *Nature 333*:801-802 (1988)). Portions of the claimed DNA sequence may also be used as probes for determining the level of expression. Similarly the protein may be used to generate antibodies that can be used in assaying cellular expression.

Portions of the *mICE2*, *Ich-1*, and *Ice-4* genes described above may be used for determining the level of expression of the proteins (*mICE2* in thymus or placental cells as well as in other tissues and organs). Such methods may be useful in determining if these cells have undergone a neoplastic transformation. Probes based upon the gene sequences may be used to isolate similar genes involved in cell death. A portion of the gene may be used in homologous recombination experiments to repair defective genes in cells or, alternatively, to develop strains of mice that are deficient in the gene. Antisense constructs may be transfected into cells (placental or thymus cells for *mICE2*) in order to develop cells which may be maintained in culture for an extended period of time or indefinitely. Alternatively antisense constructs may be used in cell culture or *in vivo* to block cell death.

The protein may be used for the purpose of generating polyclonal or monoclonal antibodies using standard techniques well known in the art (Klein, J. *Immunology: The Science of Cell-Noncell Discrimination*, John Wiley & Sons, N.Y. (1982); Kennett *et al.*, *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, N.Y. (1980); Campbell, A., "Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology 13*, Burdon *et al.* eds., Elseiver, Amsterdam (1984); Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1988)). Such antibodies may be used in assays for determining the expression of the genes. Purified protein would serve as the standard in such assays.

Based upon the sequences of Figures 6, probes may be used to determine whether the *mICE2* gene or homologs of *mICE2* are being expressed in cells. Such probes may be utilized in assays for correlating *mICE2* expression with cellular conditions, e.g. neoplastic transformation, as well as for the purpose of isolating other genes which are homologous to *mICE2*.

mICE2 will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. The diseases and conditions which could potentially be treated include neural and muscular

degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging.

Antisense nucleic acids based upon the sequences shown in Figure 6 may be used to inhibit *mICE2* expression. Such inhibition will be useful in blocking cell death in cultured cells.

The *mICE2* protein may be used to generate polyclonal or monoclonal antibodies using methods well known in the art (Klein, J. *Immunology: The Science of Cell-Noncell Discrimination*, John Wiley & Sons, N.Y. (1982); Kennett *et al., Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, N.Y. (1980); Campbell, A., "Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology 13*, Burdon *et al.* eds., Elseiver, Amsterdam (1984); Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1988)). The antibodies may be used in assays for determining the expression of *mICE2*. Purified *mICE2* protein would serve as the standard in such assays.

Based upon the sequences of Figures 8, 12A, and 12B, probes may be used to determine whether the *Ich-1* gene or homologs of *Ich-1* are being expressed in cells. Such probes may be utilized in assays for correlating *Ich-1* expression with cellular conditions, e.g. neoplastic transformation, as well as for the purpose of isolating other genes which are homologous to *Ich-1*.

Ich-1 will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. The diseases and conditions which could potentially be treated include neural and muscular degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging.

Antisense nucleic acids based upon the sequences shown in Figures 8, 12A, and 12B, may be used to inhibit *Ich-1* expression. Such inhibition will be useful in blocking cell death in cultured cells.

The *Ich-1* protein may be used to generate polyclonal or monoclonal antibodies using methods well known in the art (Klein, J. *Immunology: The*

Science of Cell-Noncell Discrimination, John Wiley & Sons, N.Y. (1982); Kennett *et al.*, *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, N.Y. (1980); Campbell, A., "Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology 13*, Burdon *et al.* eds., Elseiver, Amsterdam (1984); Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1988)). The antibodies may be used in assays for determining the expression of *Ich-1*. Purified *Ich-1* protein would serve as the standard in such assays.

Based upon the sequence of Figure 16, probes may be used to determine whether the *Ice-4* gene or homologs of *Ice-4* are being expressed in cells. Such probes may be utilized in assays for correlating *Ice-4* expression with cellular conditions, e.g. neoplastic transformation, as well as for the purpose of isolating other genes which are homologous to *Ice-4*.

Ice-4 will be used in the development of therapeutic methods for diseases and conditions characterized by cell death. The diseases and conditions which could potentially be treated include neural and muscular degenerative diseases, myocardial infarction, stroke, virally induced cell death and aging.

Antisense nucleic acids based upon the sequence shown in Figure 16 may be used to inhibit *Ice-4* expression. Such inhibition will be useful in blocking cell death in cultured cells.

The *Ice-4* protein may be used to generate polyclonal or monoclonal antibodies using methods well known in the art (Klein, J. *Immunology: The Science of Cell-Noncell Discrimination*, John Wiley & Sons, N.Y. (1982); Kennett *et al.*, *Monoclonal Antibodies, Hybridoma: A New Dimension in Biological Analyses*, Plenum Press, N.Y. (1980); Campbell, A., "Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology 13*, Burdon *et al.* eds., Elseiver, Amsterdam (1984); Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y. (1988)). The antibodies may be used in assays for

determining the expression of *Ice-4*. Purified *Ice-4* protein would serve as the standard in such assays.

Method for Preventing Programmed Cell Death in Vertebrate Cells by Inhibiting the Enzymatic Activity of Interleukin-1β Converting Enzyme (ICE)

The present invention is directed to preventing the programmed death of vertebrate cells by inhibiting the action of *ICE*. The detailed structural analysis performed on the *ced-3* gene from *C. elegans* revealed a homology to human and murine *ICE* which is especially strong at the QACRG active domain of *ICE* (see Figure 3A). *ICE* is a cysteine protease that cleaves inactive pro-interleukin-β into active interleukin-1β.

In order to determine if *ICE* functions as a cell death gene in vertebrates, the mouse *ICE* gene was cloned, inserted into an expression vector and then transfected into rat cells. A close correlation was found between *ICE* expression and cell death (see Example 2).

Further support for the function of *ICE* as a cell death gene was obtained from inhibition studies. The cowpox gene *crmA* encodes a protein that specifically inhibits *ICE* activity (Ray *et al.*, *Cell* 69:597-604 (1992)). In order to determine whether cell death can be prevented by inhibiting the enzymatic action of *ICE*, cell lines were established which produced a high level of *crmA* protein. When these cells were transfected with *ICE*, it was found that a large percentage of the cells expressing *ICE* maintained a healthy morphology and did not undergo programmed cell death.

Evidence that *ICE* has a physiological role as a vertebrate cell death gene was also obtained by examining cells engineered to over-express *bcl-2*, an oncogene known to inhibit programmed cell death and to be overexpressed in many follicular and B cell lymphomas. It was found that cells expressing *bcl-2* did not undergo cell death despite the synthesis of high levels of *ICE*. These results suggest that *bcl-2* may promote malignancy by inhibiting the action of *ICE*.

Any method of specifically regulating the action of *ICE* in order to control programmed cell death in vertebrates is encompassed by the present invention. This would include not only inhibitors specific to *ICE*, e.g. *crmA*, or the inhibitors described by Thornberry *et al.*, *Nature 356*:768-774 (1992), but also any method which specifically prevented the expression of the *ICE* gene. Thus, antisense RNA or DNA comprised of nucleotide sequences complementary to *ICE* and capable of inhibiting the transcription or translation of *ICE* are within the scope of the invention (see Lichtenstein, C., *Nature 333*:801-802 (1988)).

The ability to prevent vertebrate programmed cell death is of use in developing cells which can be maintained for an indefinite period of time in culture. For example, cells over-expressing *crmA* may be used as hosts for expressing recombinant proteins. The ability to prevent programmed cell death may allow cells to live independent of normally required growth factors. It has been found that microinjecting *crmA* mRNA or a *crmA*-expressing nucleic acid construct into cells allows chicken sympathetic neurons to live *in vitro* after the removal of neural growth factor.

Alternatively, the expression of *ICE* may be increased in order to cause programmed cell death. For example, homologous recombination may be used to replace a defective region of an *ICE* gene with its normal counterpart. In this way, it may be possible to prevent the uncontrolled growth of certain malignant cells. Methods of increasing *ICE* activity may be used to kill undesired organisms such as parasites. *crmA* is a viral protein which is important for cowpox infection. This suggests that the prevention of cell death may be important for successful infection and that, by the promotion of *ICE* expression, may provide a means for blocking infection.

Having now generally described this invention, the same will be further described by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless

otherwise specified. All references cited throughout the specification are incorporated by reference in their entirety.

Example 1

Experimental Procedures

General Methods and Strains

The techniques used for culturing *C. elegans* have been described by Brenner (Brenner, S., *Genetics 77*:71-94 (1974)). All strains were grown at 20°C. The wild-type parent strains were *C. elegans* variety Bristol strain N2, Bergerac strain EM1002 (Emmons *et al., Cell 32*:55-65 (1983)), *C. briggsae* and *C. vulgaris*. The genetic markers used are described below. These markers have been previously described (Brenner, S., *Genetics 77*:71-94 (1974)); and Hodgkin *et al., Genetics in the Nematode Caenorhabditis Elgens* (Wood *et al.* eds.) pp.491-584, Cold Spring Harbor, New York (1988)). Genetic nomenclature follows the standard system (Horvitz *et al., Mol. Gen. Genet. 175*:129-133 (1979)).

LG I: *ced-1 (ei 735); unc-54 (r323)*

LG VI: *unc-31 (e928), unc-30 (e191), ced-3 (n717, n718, n1040, n1129, n11634, n1164, n1165, n1286, n1949, n2426, n2430, n2433), unc-26 (e205), dpy-4 (e1166)*

LG V: *eg-1(n986); unc-76 (e911)*

LG X: *dpy-3(e27)*

Isolation of additional alleles of ced-3

A non-complementation screen was designed to isolate new alleles of *ced-3*. Because animals heterozygous for *ced3(n717)* in trans to a deficiency are viable (Ellis *et al., Cell 44*:817-829 (1986)), it was expected that animals

carrying a complete loss-of-function mutant *ced-3* allele in trans to *ced-3(n717)* would be viable even if homozygotes for the allele were inviable. EMS mutagenized *egl-1* L4 males were mated with *ced-3(n717) unc-26(e205)*; *egl-1(n487)*; *dpy-3(e27)* hermaphrodites. *egl-1* was used as a marker in this screen. Dominant mutations in *egl-1* cause the two hermaphrodite-specific neurons, the HSNs, to undergo programmed cell death (Trent *et al.*, *Genetics 104*:619-647 (1983)). The HSNs are required for normal egg-laying, and *egl-1(n986)* hermaphrodites, which lack HSNs are egg-laying defective (Trent *et al.*, *Genetics 104*:619-647)). The mutant phenotype of *egl-1* is suppressed in a *ced-3*; *egl-1* strain because mutations in *ced-3* block programmed cell deaths. *egl-1* males were mutagenized with EMS and crossed with *ced3(n717) unc-26(e205)*; *egl-1(n487)*; *dpy-3(e27)*. Most cross progeny were egg-laying defective because they were heterozygous for *ced-3* and homozygous for *egl-1*. Rare egglaying competent animals were picked, those animals being candidates for carrying new alleles of *ced-3*. Four such animals were isolated from about 10,000 Fl cross progeny of EMS-mutagenized animals. These new mutations were made homozygous to confirm that they carried mutations of *ced-3*.

RFLP mapping

Two cosmid libraries were used extensively in this work - a *Sau3A* I partial digest genomic library of 7000 clones in the vector pHC79 and a *Sau3A* I partial digest genomic library of 6000 clones in the vector pJB8 (Coulson *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 83*:7821-7825 (1986).

Bristol (N2) and Bergerac (EM1002) DNA was digested with various restriction enzymes and probed with different cosmids to look for RFLPs. *nP33* is a *Hind*III RFLP detected by the "right" end of Jc8. The "right" end of Jc8 was made by digesting Jc8 with *Eco*RI and self-ligating. *nP34* is a *Hind*III RFLP detected by the "left" end of Jc8. The "left" end of Jc8 was made by digesting Jc8 by *Sal*I and self ligating. *nP36* and *nP37* are both *Hind*III RFLPs detected by T10H5 and B0564, respectively.

Germ line transformation

The procedure used for microinjection basically follows that of A. Fire (Fire, A., *EMBO J.* 5:2673-2680 (1986)). Cosmid DNA was twice CsCl gradient purified. Miniprep DNA was used when deleted cosmids were injected and was prepared from 1.5 ml overnight bacteria culture in superbroth. Superbroth was prepared by combining 12 g Bacto tryptone, 24 g yeast extract, 8 ml 50% glycerol and 900 ml H_2O. The mixture was autoclaved and then 100 ml of 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4 were added. The bacterial culture was extracted by the alkaline lysis method as described in Maniatis et al. (*Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press (1983)). DNA was treated with RNase A (37°, 30 min) and then with protease K (55°, 30 min). The preparation was phenol- and then chloroform-extracted, precipitated twice (first in 0.3 M Na acetate and second in 0.1 M K acetate, pH 7.2), and resuspended in 5 l injection buffer as described by A. Fire (Fire, A., *EMBO J.* 5:2673-2680 (1986)). The DNA concentration for injection was in the range of 100 μg to 1 mg per ml.

All transformation experiments used the *ced-1(e1735)*; *unc-31(e928) ced-3(n717)* strain. *unc-31* was used as a marker for co-transformation (Kim *et al.*, *Genes & Dev.* 4:357-371 (1990)). *ced-1* was present to facilitate scoring of the *ced-3* phenotype. The mutations in *ced-1* block the engulfment process of cell death, which makes the corpses of the dead cells persist much longer than that in the wild-type (Hedgecock *et al.*, *Science 220*:1277-1280 (1983)). *ced-3* phenotype was scored as the number of dead cells present in the head of young L1 animals. The cosmid C10D8 or the plasmid subclones of C10D8 were mixed with C14G10 (*unc-31(+)*-containing) at a ratio of 2:1 or 3:1 to increase the chances that an Unc-31(+) transformant would contain the cosmid or plasmid being tested. Usually, 20-30 animals were injected in one experiment. Non-Unc F1 progeny of injected animals were isolated three to four days later. About 1/2 to 1/3 of the non-Unc progeny transmitted the non-Unc phenotype to F2 and established a line of transfomants. The young

L1 progeny of such non-Unc transformants were checked for the number of dead cells present in the head using Nomarski optics.

Determination of ced-3 transcript initiation site.

Two primers, Pex1: (5'GTTGCACTGCTTTCACGATCTCCCGTCTCT3') and Pex2: (5'TCATCGACTTTTAGATGACTAGAGAACATC3'), were used for primer extension. The primers for RT-PCR are: SL1 (5'GTTTAATTACCCAAGTTTGAG3') and log-5 (5'CCGGTGACATTGGACACTC3'). The products are reamplified using the primers SL1 and oligo10 (5'ACTATTCAACACTTG3'). A product of the expected length was cloned into the PCR1000 vector (invitrogen) and sequenced.

Determination and analysis of DNA sequence

For DNA sequencing, serial deletions were made according to a procedure developed by Henikoff (Heinkoff, S., *Gene 28*:351-359 (1984)). DNA sequences were determined using Sequenase and protocols obtained from US Biochemicals with minor modifications.

The *ced-3* amino acid sequence was compared with amino acid sequences in the GenBank, PIR and SWISS-PROT databases at the National Center for Biotechnology Information (NCBI) using the blast network service.

Cloning of ced-3 genes from other nematode species

C. briggsae and *C. vulgaris ced-3* genes were isolated from corresponding phage genomic libraries using the *ced-3* cDNA subclone pJ118 insert as a probe under low stringency conditions (5xSSPE, 20% Formamide, 0.02% Ficoll, 0.02% BSA, 0.02% polyvinylpyrrolidone, 1% SDS) at 40°C

overnight and washed in 1xSSPE and 0.5% SDS twice at room temperature and twice at 42°C for 20 min each time.

Results

ced-3 is not essential for viability

All previously described *ced-3* alleles were isolated in screens designed to detect viable mutants in which programmed cell death did not occur (Ellis *et al., Cell 44*:817-829 (1986)). Such screens might systematically have missed classes of *ced-3* mutations that result in inviability. Since animals with the genotype of *ced-3*/deficiency are viable (Ellis *et al., Cell 44*:817-829 (1986)), a noncomplementation-screening scheme was designed that would allow the isolation of recessive lethal alleles of *ced-3*. Four new *ced-3* alleles (*n1163, n1164, n1165,* and *n1286*) were obtained which were viable as homozygotes. These new alleles were isolated at a frequency of about 1 in 2500 mutagenized haploid genomes, approximately the frequency expected for the generation of null mutations in an average *C. elegans* gene (Brenner, S., *Genetics 77*:71-94 (1974); Meneely *et al., Genetics 92*:99-105 (1990); Greenwald *et al., Genetics 96*:147-160 (1980)).

These results suggest that animals that lack *ced-3* gene activity are viable. In support of this hypothesis, molecular analysis has revealed that three *ced-3* mutations are nonsense mutations that prematurely terminate *ced-3* protein translation and one alters a highly conserved splice acceptor site (see below). These mutations would be expected to eliminate *ced-3* activity completely. Based upon these considerations, it was concluded that *ced-3* gene activity is not essential for viability.

ced-3 is contained within a 7.5 kb genomic fragment

The *ced-3* gene was cloned using the approach of Ruvkun *et al.* (*Molecular Genetics of the Caenorhabditis Elgens Heterochronic Gene lin-14* *121*:501-516 (1988)). Briefly (for further details, see Experimental Procedures), the *C. elegans* Bristol strain N2 contains 30 dispersed copies of the transposable element Tc1, whereas the Bergerac strain contains more than 400 copies (Emmons *et al.*, *Cell 32*:55-65 (1983); Finney, M., Ph.D. Thesis "The Genetics and Molecular Biology of *unc-86*, a *Caenorhabditis elgens* Cell Lineage Gene," Cambridge, MA (1987)). By crossing Bristol and Bergerac strains, a series of recombinant inbred strains were generated in which chromosomal material was mostly derived from the Bristol strain with varying amounts of Bergerac-specific chromosome IV-derived material in the region of the *ced-3* gene. By probing DNA from these strains with plasmid pCe2001 which contains Tc1 (Emmons *et al.*, *Cell 32*:55-65 (1983,) a 5.1 kb EcoRI Tc1-containing restriction fragment specific to the Bristol strain (restriction fragment length polymorphism nP35) and closely linked to *ced-3* was identified.

Cosmids that contained this 5.1 kb restriction fragment were identified and it was found that these cosmids overlapped an existing cosmid contig that had been defined as part of the *C. elegans* genome project (Coulson *et al.*, *Proc. Natl. Acad. Sci. 83*:7821-7825 (1986). Four other Bristol-Bergerac restriction fragment length polymorphisms were defined by cosmids in this contig (*nP33, np34, nP36, nP37*). By mapping these restriction fragment length polymorphisms with respect to the genes *unc-30*, *ced-3* and *unc-26*, the physical contig was oriented with respect to the genetic map and the region containing the *ced-3* gene was narrowed to an interval spanned by three cosmids (Fig. 1). By mapping these RFLPs between Bristol and Bergerac strains with respect to the genes *unc-30*, *ced-3* and *unc-26*, the physical contig was oriented with respect to the genetic map.

On Southern blot, three of three + Berg *unc-26* recombinants showed the Bristol nP33 pattern while two of two *ced-3* + Berg recombinants showed the Bergerac pattern (data not shown). Thus, nP33 maps very close or to the right of *unc-26*. For nP34, two of two *ced-3* + Berg recombinants and two of three + Berg *unc-26* recombinants showed the Bergerac pattern; one of the three + Berg *unc-26* recombinant showed the Bristol pattern (data not shown). The genetic distance between *ced-3* and *unc-26* is about 0.2 mu. Thus, nP34 maps between *ced-3* and *unc-26*, about 0.1 mu to the right of *ced-3*. Similar experiments mapped nP35, the 5.1 kb Bristol specific Tc1 element, to about 0.1 mu to the right of *ced-3* (data not shown).

In order to map *n36* and *n37*, Bristol *unc-30 ced-3*/++ males were crossed with Bergerac hermaphrodites. From the progeny of heterozygotes of genotype *unc-30 ced-3* (Bristol)/++ (Bergerac), Unc-30 non-*ced-3* and non-Unc-30 *ced-3* animals were picked and DNA was prepared from these strains. *nP36* maps very close or to the right of *unc-30* since two of two *unc-30* + Berg recombinants showed Bristol pattern and two of two + Berg *ced-3* recombinants showed the Bergerac pattern (data not shown). Similarly, nP37 maps very close or to the right of *unc-30* since four of the four + Berg *ced-3* showed Bergerac pattern and six of six *unc-30* + Berg recombinants showed the Bristol pattern (data not shown). These experiments narrowed the region containing the ced-3 gene to an interval spanned by the three cosmids (Fig. 1a).

Cosmids that were candidates for containing the *ced-3* gene were microinjected (Fire, A., *EMBO J.* 5:2673-2680 (1986)) into *ced-3* mutant animals to test for rescue of the mutant phenotype. Specifically, cosmid C14G10, which contains the wild-type *unc-31* gene and a candidate cosmid were coinjected into *ced-1(e1375)*; *unc-31(e928) ced-3(n717)* hermaphrodites. Non-unc progeny were isolated and observed to see if the non-Unc phenotype was transmitted to the next generation, thus establishing a line of transformed animals. Young L1 progeny of such transformant lines were examined for the presence of cell deaths using Nomarski optics to see whether the *ced-3*

phenotype was complemented (see Experimental Procedures). Cosmid C14G10 alone does not confer *ced-3* activity when injected into a *ced-3* mutant.

unc-31 was used as a marker for co-transformation (Kim *et al.*, *Genes & Devel. 4*:357-371 (1990)). *ced-1* was present to facilitate scoring of the *ced-3* phenotype. Mutations in *ced-1* block the engulfment process of programmed cell death, causing the corpses of dead cells to persist much longer than in the wild-type (Hedgecock *et al.*, *Science 220*:1277-1280 (1983)). Thus, the presence of a corpse indicates a cell that has undergone programmed cell death. The *ced-3* phenotype was scored as the number of corpses present in the head of young L1 animals.

As indicated in Fig. 1, of the three cosmids injected (C43C9, W07H6 and C48D1), only C48D1 rescued the *ced-3* mutant phenotype. Both non-Unc transformed lines obtained, *nIs1* and *nEx2*, were rescued. Specifically, L1 ced-1 animals contain an average of 23 cell corpses in the head, and L1 ced-1; ced3 animals contain an average of 0.3 cell corpses in the head (Ellis *et al.*, *Cell 44*:817-829 (1986)). By contrast, *ced-1*; *unc-31 ced-3*; *nIs1*; and *ced-1*; *unc-31 ced-3*; *nEx2* animals contained an average of 16.4 and 14.5 cell corpses in the head, respectively. From these results, it was concluded that C48D1 contains the *ced-3* gene.

To locate *ced-3* more precisely within the cosmid C48D1, this cosmid was subcloned and the subclones tested for their ability to rescue the *ced-3* mutant phenotype (Fig. 1A). From these experiments, *ced-3* was localized to a DNA fragment of 7.5 kb (pJ7.5).

A 2.8 kb ced-3 transcript is expressed primarily during embryogenesis and independently of ced-4 function

The 7.6 kb pJ107 subclone of C48D1 (Fig. 1A) was used as a probe in a northern blot of polyA+ RNA derived from the wild-type *C. elegans* strain N2. This probe hybridized to a 2.8 kb transcript. Although this transcript is present in 11 different EMS-induced *ced-3* mutant strains,

subsequent analysis has shown that all 11 tested *ced-3* mutant alleles contain mutations in the genomic DNA that encodes this mRNA (see below), thus establishing this RNA as a *ced-3* transcript.

The developmental expression pattern of *ced-3* was determined by hybridizing a northern blot of RNA from animals at different stages of development with the *ced-3* cDNA subclone pJ118 (see below). The *ced-3* transcript was found to be most abundant during embryogenesis, when most programmed cell deaths occur, but was also detected during the L1 through L4 larval stages. It is present in relatively high levels in young adults.

Since *ced-3* and *ced-4* are both required for programmed cell death in *C. elegans*, and since both are highly expressed during embryonic development (Yuan *et al., Dev. 116*:309-320 (1992), the possibility existed that one of the genes might regulate the mRNA level of the other. Previous studies have revealed that *ced-3* does not regulate *ced-4* mRNA levels (Yuan *et al., Dev. 116*:309-320 (1992)). To determine if *ced-4* regulates *ced-3* mRNA levels, a northern blot of RNA prepared from *ced-4* mutant embryos was probed with the *ced-3* cDNA subclone pJ118. It was found that the amount and size of the *ced-3* transcript was normal in the *ced-4* mutants ***n1162, n1416, n1894 and n1920.*** Thus, *ced-4* does not appear to affect the steady-state levels of *ced-3* mRNA.

ced-3 cDNA and Genomic Sequences

To isolate *ced-3* cDNA clones, *ced-3* genomic DNA pJ40 (Fig. 1A) was used as a probe to screen a cDNA library of the *C. elegans* wild-type strain N2 (Kim *et al., Genes & Dev. 4*:357-371 (1990)). The 2.5 kb cDNA clone pJ87 was isolated in this way. On northern blots, pJ87 hybridized to a 2.8 kb transcript and on Southern blots, it hybridized only to bands to which pJ40 hybridizes (data not shown). Thus, pJ87 represents an mRNA transcribed entirely from pJ40 which can rescue the *ced-3* mutant phenotype when microinjected into *ced-3* mutant animals. To confirm that pJ87 contains

the *ced-3* cDNA, a frameshift mutation in the *Sal*I site of pJ40 was made corresponding to the *Sal*I site in the pJ87 cDNA. Constructs containing the frameshift mutation failed to rescue the *ced-3* phenotype when microinjected into *ced-3* mutant animals (6 transformant lines; data not shown), suggesting that *ced-3* activity had been eliminated by mutagenizing a region of genomic DNA that corresponds to the pJ87 cDNA.

The DNA sequence of pJ87 is shown in Figure 2C. pJ87 contains an insert of 2482 bp with an open reading frame of 503 amino acids. It has 953 bp of 3′ untranslated sequence, not all of which is essential for *ced-3* expression; genomic constructs that do not contain 380 bp of the 3′-most region (pJ107 and its derivatives, see Fig. 1a) were capable of rescuing *ced-3* mutant phenotype. The cDNA ends with a poly-A sequence, suggesting that the complete 3′ end of the *ced-3* transcript is present.

To confirm the DNA sequence obtained from the *ced-3* cDNA and to study the structure of the *ced-3* gene, the genomic sequence of the *ced-3* gene from the plasmid pJ107 was determined. The insert in pJ107 is 7656 bp in length (Fig. 2).

To determine the location and nature of the 5' end of the *ced-3* transcript, a combination of primer extension and amplification using the polymerase chain reaction (PCR) was used. Two primers, Pex1 and Pex2, were used for primer extension. The Pex1 reaction yielded two major bands, whereas the Pex2 reaction gave one band. The Pex2 band corresponds in size to the smaller band from the Pex1 reaction, and agrees in length with a possible transcript that is trans-spliced to a *C. elegans* splice leader (Bektesh *et al., Genes and Dev. 2*:1277-1283 (1988)) at a consensus splice acceptor at position 2166 of the genomic sequence. The nature of the larger Pex1 band is unclear.

To confirm these observations, wild-type total RNA was reverse-transcribed and then amplified using the primers SL1 and log-5 followed by reamplification using the primers SL1 and oligol0. A product of the expected length was cloned into the PCR1000 vector (invitrogen) and sequenced. The

sequence obtained confirmed the presence of a *ced-3* message trans-spliced to SL1 at position 2166 of the genomic sequence. These experiments suggest that a *ced-3* transcript is trans-spliced to the *C. elegans* splice leader SL1 (Bektesh *et al., Genes and Dev. 2*:1277-1283 (1988)) at a consensus splice acceptor at position 2166 of the genomic sequence. Based upon these observations, it is concluded that the start codon of *ced-3* protein is the methionine encoded at position 2232 of the genomic sequence and that the *ced-3* protein is 503 amino acids in length.

The predicted *ced-3* protein is hydrophilic (256/503 residues are charged or polar) and does not contain any obvious potential trans-membrane domains. One region of the *ced-3* protein is rich in serines: from amino acid 107 to amino acid 205, 32 of 99 amino acids are serine residues.

The sequences of 12 EMS-induced *ced-3* mutations (Table 1) were determined. Eight are missense mutations, three are nonsense mutations, and one alters a conserved G at the splice acceptor site of intron 6. Interestingly, nine of these 12 mutations alter residues within the last 100 amino acids of the protein, and none occurs within the serine-rich region.

Table 1. Sites of mutations in the ced-3 gene.

Allele	Mutation	Nucleotide	Codon	Consequence
n717	G to A	6297		Altered splicing
n718	G to A	2487	65	G to R
n1040	C to T	2310	27	L to F
n1129 & n164	C to T	6434	449	A to V
n1163	C to T	7020	486	S to F
n1165	C to T	5940	403	Nonsense
n1286	G to A	6371	428	Nonsense
n1949	C to T	6222	412	Nonsense
n2426	G to A	6535	483	E to K
n2430	C to T	6485	466	A to V
n2433	G to A	5757	360	G to S

Nucleotide and codon positions correspond to the numbering in Fig. 2.

To identify functionally important regions of the *ced-3* protein, the genomic sequences of the *ced-3* genes from the related nematode species *C. briggsae* and *C. vulgaris* were cloned and sequenced. Sequence comparison of the three *ced-3* genes showed that the relatively non-serine-rich regions of the proteins are more conserved than are serine-rich regions (Fig. 3A). All 12 EMS-induced *ced-3* mutations altered residues that are conserved among the three species. These results suggest that the non-serine-rich region is important for *ced-3* function and that the serine rich region is either unimportant or that residues within it are functionally redundant.

ced-3 protein is similar to the mammalian ICE and Nedd-2 proteins

A search of the GenBank, PIR and SWISS-PROT databases revealed that the non-serine-rich regions of the *ced-3* protein are similar to human and

murine interleukin-1β (IL-1β) convertases (*ICE*) (Fig. 3A). *ICE* is a cysteine protease that cleaves the inactive 31 KD precursor of IL-1β between Asp^{116} and Ala^{117} releasing a carboxy-terminal 153 amino-acid peptide known as mature IL-1β (Kostura *et al., Proc. Natl. Acad. Sci., USA 86*:5227-5231 (1989); Black *et al., FEBS Lett. 247*:386-390 (1989)). The most highly conserved region among the proteins shown in Figure 3A consists of amino acids 246-360 of the *ced-3* protein and amino acids 166-287 of the human *ICE* protein: 49 residues are identical (43% identity). The active site cysteine of human *ICE* is located at cysteine 285 (Thornberry *et al., Nature 356*:768-774 (1992)). The five-amino-acid peptide (QACRG) around this active cysteine is the longest conserved peptide among the murine and human *ICE* proteins and *ced-3* proteins from nematodes.

Human *ICE* is composed of two subunits (p20 and p10) that appear to be proteolytically cleaved from a single proenzyme by the mature enzyme (Thornberry *et al., Nature 356*:768-774 (1992)). Two cleavage sites in the proenzyme, Asp-Ser at positions 103 and 297 of *ICE*, are conserved in *ced-3* (position 131 and 371, respectively).

The C-terminal portion of the *ced-3* protein and the p10 subunit of *ICE* are similar to the protein product of the murine *nedd-2* gene, which is highly expressed during embryonic brain development and is down-regulated in adult brain (Kumar *et al., Biochem and Biophy. Res. Comm. 185*:1155-1161 (1992)). The *ced-3* and *nedd-2* proteins, and the *ICE* and *nedd-2* proteins are 27% identical (Fig. 3A). The *nedd-2* protein does not contain the QACRG peptide at the active site of *ICE* (Fig. 3A). Seven of eight point mutations that were analyzed (*n718, n1040, n1129, n1164, n2430, n2426 & n2433*) result in alterations of amino acids that are conserved or semi-conserved among the three nematode *ced-3* proteins, *ICE* and the *nedd-2* protein. In particular, the mutation, n2433, introduces a Gly to Ser change near the putative active cysteine (Fig. 2, Table 1).

Discussion

The genes *ced-3* and *ced-4* are the only genes known to be required for programmed cell death to occur in *C. elegans* (Ellis *et al., Cell 44*:817-829 (1986)). Genetic and molecular studies have revealed that the *ced-3* gene shares a number of features with *ced-4*. Like *ced-4* (see Yuan *et al., Dev. 116*:309-320 (1992)), *ced-3* is not required for viability. It appears to encode a single mRNA which is expressed mostly in the embryo, the stage during which 113 of the 131 programmed cell death occur. Furthermore, just as *ced-3* gene function is not required for *ced-4* gene expression (Yuan *et al., Dev. 116*:309-320 (1992)), *ced-4* gene function is not required for *ced-3* gene expression. Thus, these two genes do not appear to control the onset of programmed cell death by acting sequentially in a transcriptional regulatory cascade. Unlike *ced-4* (Yuan *et al., Dev. Biol. 138*:33-41 (1992)), *ced-3* is expressed at a substantial level in young adults, this observation suggests that *ced-3* expression might not be limited to cells undergoing programmed cell death.

The *ced-4* protein is novel in sequence, and the only hint concerning its function is that two regions of the protein show some similarity to the EF-hand motif, which binds calcium (Yuan *et al., Dev. 116*:309-320 (1992)). For this reason it has been suggested that the *ced-4* protein and hence programmed cell death in *C. elegans* might be regulated by calcium. However, no direct evidence for this hypothesis has yet been obtained. The *ced-3* protein similarly contains a region that offers a clue about possible biochemical function: a region of 99 amino acids contains 32 serines. Since serines are common phosphorylation sites (Edelman *et al., Ann. Rev. Biochem. 56*:567-613 (1987)), it is possible that the *ced-3* protein and hence programmed cell death in *C. elegans* is regulated by phosphorylation. Phosphorylation has previously been suggested to function in cell death. McConkey *et al.* (McConkey *et al., J. Immunol. 145*:1227-1230 (1990)) have shown that several agents that can elevate cytosolic cAMP level induce thymocyte death,

suggesting that protein kinase A may mediate cell death by phosphorylating certain proteins. Although the precise sequence of the serine-rich region varies among the three *Caenorhabditis* species studied, the relatively high number of serines is conserved in *C. elegans*, *C. briggsae* and *C. vulgaris*. None of the mutations in *ced-3* affect the serine-rich region. These observations are consistent with the hypothesis that the presence of serines is more important than the precise amino acid sequence within this region.

Much more striking than the presence of the serine-rich region in the *ced-3* protein is the similarity between the non-serine-rich regions of *ced-3* and the human and murine interleukin-1β converting enzyme (*ICE*). Human *ICE* is a substrate-specific protease that cleaves 31 KD prointerleukin-1β at Asp^{116}-Ala^{117} to produce the mature 17.5 kD interleukin-1β (IL-1β). IL-1β is a cytokine involved in mediating a wide range of biological responses including inflammation, septic shock, wound healing, hematopoiesis and growth of certain leukemias (Dinarello, C.A., *Blood 77*:1627-1652 (1991); diGiovine *et al., Today 11*:13 (1990)). A specific inhibitor of *ICE*, the *crmA* gene product of Cowpox virus, prevents the proteolytic activation of interleukin-1β (Ray *et al., Cell 69*:597-604 (1992)) and inhibits host inflammatory response (Ray *et al., Cell 69*:597-604 (1992)). Cowpox virus carrying a deleted *crmA* gene is unable to suppress the inflammatory response of chick embryos, resulting in a reduction in the number of virus-infected cells and less damage to the host (Palumbo *et al., Virology 171*:262-273 (1989)). This observation indicates the importance of *ICE* in bringing about the inflammatory response.

The carboxy half of the *ced-3* protein is the region most similar to *ICE*. A stretch of 115 residues (amino acids 246-360 of *ced-3*) is 43% identical between the *ced-3* and *ICE* proteins. This region contains a conserved pentapeptide QACRG (positions 361-365 of the *ced-3* protein), which surrounds the active cysteine of *ICE*. Specific modification of this cysteine in human *ICE* results in complete loss of activity (Thronberry *et al., Nature 356*:768-774 (1992)). The *ced-3* mutation *n2433* alters the conserved glycine in this pentapeptide and eliminates *ced-3* function, suggesting that this glycine

is important for *ced-3* activity and might be an integral part of the active site of *ICE*. Interestingly, the mutations *n718* (position 67 of *ced-3*) and *n1040* (position 27 of *ced-3*) eliminate *ced-3* function *in vivo* yet they contain alterations in conserved residues which are outside of mature P20 subunit of *ICE* (Thronberry *et al., Nature* 356:768-774 (1992)). Perhaps these residues have a non-catalytic role in both *ced-3* and *ICE* function, e.g. they may maintain a proper conformation for proteolytic activation. The *ICE* precursor (p45) is proteolytically cleaved at 4 sites of *ICE* (Asp103, Asp119, Asp297 and Asp316) to generate p24, p20, and p10 (Thronberry *et al., Nature* 356:768-774 (1992)). At least two of the cleavage sites are conserved in *ced-3* indicating that the *ced-3* product might be processed as well.

The similarity between the *ced-3* and *ICE* proteins strongly suggests that *ced-3* might function as a cysteine protease in controlling programmed cell death by proteolytically activating or inactivating a substrate protein. One potential substrate for *ced-3* might be the product of the *ced-4* gene which contains 6 Asp residues that might be the target of *ced-3* protein (Asp25, Asp151, Asp185, Asp192, Asp459 and Asp541). Alternatively, the *ced-3* protein might directly cause cell death by proteolytically cleaving certain proteins or subcellular structures that are crucial for cell viability.

ced-3 and *ICE* are part of a novel protein family. Thornberry *et al.* suggested that the sequence GDSPG at position 287 of *ICE* resembles a GX(S/C)XG motif found in serine and cysteine protease active sites (*Nature* 356:768-774 (1992)). However, in the three nematode *ced-3* proteins examined, only the first glycine of this sequence is conserved and in mouse *ICE* the S/C is missing. This suggests that the *ced-3/ICE* family shares little sequence similarity with known protease families.

The similarity between *ced-3* and *ICE* suggests, not only that *ced-3* functions as a cysteine protease, but also that *ICE* functions in programmed cell death in vertebrates. Consistent with this hypothesis, it has been observed that after murine peritoneal macrophages are stimulated with lipopolysaccharide (LPS) and induced to undergo programmed cell death by

exposure to extracellular ATP, mature active IL-1β is released into the culture supernatant. In contrast, when cells are injured by scraping, IL-1β is released exclusively as the inactive proform (Hogoquist *et al., Proc. Natl. Acad. USA 88*:8485-8489 (1991)). These results suggest that *ICE* is activated upon induction of programmed cell death. *ICE* transcript has been detected in cells that do not make IL-1β (Cerretti *et al., Science 256*:97-100 (1992)), suggesting that other *ICE* substrates exist. This suggests that *ICE* could mediate programmed cell death by cleaving a substrate other than IL-1β.

The carboxy-terminal portions of both the *ced-3* protein and the p10 subunit of *ICE* are similar to the protein encoded by the murine *nedd-2* gene, which is expressed preferentially during early embryonic brain development (Kumar *et al., Biochem and Biophy. Res. Comm. 185*:1155-1161 (1992)). Since the *nedd-2* protein lacks the QACRG active domain, it might function to regulate an *ICE* or *ICE*-like p20 subunits. Interestingly, four *ced-3* mutations alter residues conserved between the *nedd-2* and *ced-3* proteins and *nedd-2* gene expression is high during embryonic brain development, when much programmed cell death occurs. These observations suggest that *nedd-2* might function in programmed cell death.

The *C. elegans* gene *ced-9* protects cells from undergoing programmed cell death by directly or indirectly antagonizing the activities of *ced-3* and *ced-4* (Hengartner *et al., Nature 356*:494-499 (1992)). The vertebrate gene *bcl-2* acts in a way functionally similar to *ced-9*. Overexpression of *bcl-2* protects or delays the onset of apoptotic cell death in a variety of vertebrate cell types as well as in *C. elegans* (Vaux *et al., Science 258*:1955-1957 (1992); Nunez *et al., J. Immun. 144*:3602-3610 (1990); Vaux *et al., Science 258*:1955-1957 (1992); Sentman *et al., Cell 67*:879-888 (1992); Strasser *et al., Cell 67*:889-899 (1991)). Thus, if *ICE* or another *ced-3/ICE* family member is involved in vertebrate programmed cell death, it is possible that *bcl-2* could act by modulating its activity. The fact that *bcl-2* is a dominant oncogene (overexpression of *bcl-2* as a result of chromosomal translocation occurs in 85% of follicular and 20% of diffuse B cell lymphomas, Fukuhara *et al.*,

Cancer Res. 39:3119 (1979); Levine *et al., Blood 66*:1414 (1985); Yunis *et al., N. Engl. J. Med. 316*:79-84 (1987)) suggests that *ICE* and other *ced-3/ICE* family members might be recessive oncogenes. The elimination of such cell death genes would prevent normal cell death and promote malignancy, just as does overexpression of *bcl-2*.

Example 2

The mouse homolog of human *ICE* from a mouse thymus cDNA library (Stratagene) was cloned by low stringency hybridization using human *ICE* as a probe. This clone, named "*mICE*", is identical to the clone isolated by Net *et al.* (*J. Immun. 149*:3245-3259 (1992)) except that base pair 166 is an A and, as a result, Asn is encoded rather than Asp. This may be a DNA polymorphism since the isolated clone was from a thymus cDNA library (Stratagene) of mouse B6/CBAFlJ (C57Black x CBA) strain while Nett's clone was from a WEH13 cell cDNA library (Stratagene). Subsequent experiments have shown that this DNA polymorphism is in a region which is not essential for *ICE* function (see below). Thus, the presence of Asn rather than Asp should have no effect on the results obtained.

In order to circumvent the difficulty of establishing a permanent cell line that expresses *ICE* in high levels, a transient expression system was developed to determine if overexpression of *mICE* kills cells. *mICE* cDNA was fused with the *E. coli lac-Z* gene and the product so produced was placed under the control of chicken β-actin promoter (Fig. 4). The active *ICE* protein is known to have two subunits, P20 and PlO, which are processed from a precursor peptide (Thornberry *et al., Nature 356*:768-774 (1992)). To test the function of the subunits, two additional fusion genes were made, *P20/P10-lacZ* and *P10-lacZ*.

The constructs shown in Fig. 4 were transfected into rat 1 cells by calcium phosphate precipitation. 24 hours after transfection, cells were fixed and X-gal was added to begin the color reaction. It was found that, after 3

hours of color development, most blue cells transfected with intact *mICE-lacZ* or *P20/P10-lacZ* were round, whereas most blue cells transfected with *P10-lacZ* or the control *lac-Z* construct were normal, flat cells (Table 2). Similar results were obtained with another cell line, NG108-15 neuronal cells. Healthy living rat cells are flat and well-attached to plates whereas dying cells are round and often float into the medium.

Table 2. Overexpression of *mICE* causes rat-1 cells to undergo programmed cell death. The constructs shown in Fig. 4 are transiently transfected into rat-1 cells, rat-1 cells expressing *bcl-2* (rat-1/*bcl-2*) or rat-1 cells expressing *crmA* (rat-1/*crmA*). 24 hrs after transfection, cells are fixed and stained with X-gal for 3 hrs. The data shown are the percentage of round blue cells among total number of blue cells. The data are collected from at least three different experiments.

Construct	rat-1	rat-1/*bcl-2*	rat-1/*crmA*
pActbGal'	1.44 ± 0.18	2.22 ± 0.53	2.89 ± 0.79
pβActM10Z	80.81 ± 2.33	9.91 ± 2.08	18.83 ± 2.86
pβActM11Z	93.33 ± 2.68	13.83 ± 4.23	24.48 ± 2.78
pβactM19Z	2.18 ± 0.54	-	-
pβActM12Z	2.44 ± 0.98	3.33 ± 1.45	2.55 ± 0.32
pβact17Z	2.70 ± 1.07	-	-
pJ485	1.32 ± 0.78	-	-
pβActced38Z	46.73 ± 4.65	35.28 ± 1.36	34.40 ± 2.38
pβActced37Z	3.67 ± 1.39	-	-

Methods: a: Construction of *bcl-2* expressing vector (pJ415): pJ415 was constructed by first inserting 5', the 400bp *Bgl*II/*Bam*HI crmA fragment into the *Bam*HI site of the pBabe/puro vector and then inserting the remaining 1kb *Bam*HI crmA fragment into the 3'*Bam*HI site in the sense direction. b: Construction of the *bcl-2* expressing vector (pJ436): pJ436 was constructed by inserting an *Eco*RI/*Sal*I *bcl-2* fragment into the *Eco*RI/*Sal*I sites of the pBabe/puro vector. c: Establishing Rat-1 cell lines that overexpress *crmA* and *bcl-2*: pJ415 and pJ436 were electroporated into ΨCRE retroviral packaging cells (Danos *et al.*, Proc. Natl. Acad. Sci. U.S.A. 85:*6460-6464* (1988)) using a BioRad electroporating apparatus. Supernatant either from overnight transiently transfected ΨCRE cells or from stable lines of

ΨCRE cells expressing either *crmA* or *bcl-2* were used to infect Rat-1 cells overnight in the presence of 8 μg/ml of polybrene. Resistant cells were selected using 30 ug/ml puromycin for about 10 days. Resistant colonies were cloned and checked for expression using both Northern and Western blots. Bcl-2 antibodies were from S.J. Korsmeyer and from DAKO. *crmA* antisera was made by immunizing rabbits with anm *E. coli*-expressed *crmA* fusion protein (pJ434). pJ434 was made by inserting an *Eco*RI/*Sal*I fragment of *crmA* cDNA into *Eco*RI/*Sal*I sites of pET21a (Novagen) and fusion protein was expressed in the *E. coli* BL21 (DE3) strain. Multiple lines that express either *bcl-2* or *crmA* were checked for suppression of *mICE* induced cell death and all showed similar results.

When cells were stained with rhodamine-coupled anti-β galactosidase antibody and Hoechst dye, it was found that galactosidase-positive round cells had condensed and fragmented nuclei. Such nuclei are indicative of programmed cell death. When observed in an electron microscope, the X-gal reaction product was electron dense, allowing *ICE-lacZ* expressing cells to be distinguished from other cells (Snyder *et al.*, *Cell* 68:33-51 (1992)). The chimeric gene expressing cells showed condensed chromatin and membrane blebbing. These are characteristics of cells undergoing programmed cell death (Wyllie, A.H., in *Cell Death in Biology and Pathology*, 9-34 (1981); Oberhammer *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:5408-5412 (1992); Jacobson *et al.*, *Nature 361*:365-369 (1993)). Thus, the results indicate that overexpression of *mICE* induces programmed cell death and induction depends on both P20 and P10 subunits.

When color development in rat-1 cells transfected with *mICE-lacZ* or *P20/P10-lacZ* is allowed to proceed for 24 hours, a greater number of flat cells turn blue. This result indicates that a lower level of *ICE* activity can be tolerated by cells.

If *mICE* is a vertebrate homolog of *ced-3*, then *ced-3* might also be expected to cause cell death in vertebrates. This hypothesis was tested by making a *ced-3-lacZ* fusion construct and examining its ability to cause cell

death using the assay as described above. As expected, the expression of *ced-3* caused the death of rat cells (Table 2).

If *mICE* functions in a similar way to *ced-3*, another prediction is that mutations eliminating *ced-3* activity in *C. elegans* should also eliminate its activity in vertebrates. This hypothesis was tested by mutating the Gly residue in the pentapeptide active domain of *ICE*, QACRG, to Ser. It was found that this mutation eliminated the ability of both *mICE* and *ced-3* to cause rat cell death (Table 2).

The cowpox gene *crmA* encodes a 38 kD protein that can specifically inhibit *ICE* activity (Ray *et al., Cell 69*:597-604 (1992)). To demonstrate that cell death caused by overexpression of *mICE* is due to the enzymatic activity of *ICE* protein, rat-1 cells were infected with a pBabe retroviral construct (Morgenstern *et al., Nucl. Acids Res. 18*:3587-3596 (1990)) expressing *crmA* and cell lines were identified which produce a high level of *crmA* protein. When the *mICE-lacZ* construct was transfected into these cell lines, it was found that a large percentage of blue cells had a healthy, flat morphology (Table 2). In addition, a point mutation that changes the Cys residue in the active site pentapeptide, QACRG to a Gly eliminates the ability of *ICE* to cause cell death (construct pβactM17Z, Figure 4, Table 2). This result indicates that the proteolytic activity of *ICE* is essential to its ability to kill cells.

In mammals, *bcl-2* prevents certain cells from undergoing programmed cell death (Vaux *et al., Nature 335*:440-442 (1988); Nunez *et al., J. Immun. 144*:3602-3610 (1990); Strasser *et al., Cell 67*:889-899 (1991); Sentman *et al., Cell 67*:879-888 (1991)). Expression of *bcl-2* in the nematode *C. elegans* has been shown to partially prevent programmed cell death. Thus, *bcl-2* is functionally similar to the *C. elegans ced-9* gene (Vaux *et al., Science 258*:1955-1957 (1992); Hengartner *et al., nature 356*:494-499 (1992)).

Rat-1 cells were infected with the pBabe retroviral construct expressing *bcl-2*. Transfection of the *mICE-lacZ* fusion construct into the cells lines overexpressing *bcl-2* showed that a high percentage of blue cells were now

healthy (Table 2). Thus, cell death induced by overexpression of *mICE* can be suppressed by *bcl-2*. This result indicates that cell death induced by overexpression of *mICE* is probably caused by activation of a normal programmed cell death mechanism. The amino acid sequence of *ICE* is similar to *C. elegans ced-3*, which functions in initiating programmed cell death during development. Thus, vertebrate animals may have a genetic pathway of programmed cell death similar to that of *C. elegans* (Fig. 5).

Example 3

As described above, the genes in the *ICE/ced-3* family would be expected to function during the initiation of programmed cell death. In order to identify additional members of this gene family, cDNA encoding human interleukin-1β converting enzyme (*ICE*) was used to screen a mouse thymus cDNA library (Stratagene) under conditions of low stringency. Using this procedure, a new gene was identified and named "*mICE2*" (see Figure 6 for the cDNA sequence and deduced amino acid sequence of *mICE2*).

Figures 7 and 7A shows that the protein encoded by *mICE2* contains significant homology to both human and murine interleukin-1β converting enzyme (*ICE*), as well as to the *C. elegans* cell death gene, *ced-3*. The sequence homology indicates that *mICE2*, like *mICE*, is a vertebrate cell death gene.

Northern blot analyses showed that, unlike *mICE*, which is broadly expressed during embryonic development, the expression of *mICE2* is restricted to the thymus and placenta, areas where cell death frequently occurs. In addition, it was found that the expression of *mICE2* in the thymus can be induced by dexmethosome, an agent which causes thymus regression. It is concluded that *mICE2* is a thymus/placenta specific vertebrate cell death gene.

Example 4

Extensive cell death occurs in the developing nervous system (Oppenheim, R. W., *Ann. Rev. Neurosci. 145*:453-501 (1991)). Many neurons die during the period of synapse formation. During this critical period, the survival of neurons depends on the availability of neural trophic factors. The survival of isolated primary neurons *in vitro* depends critically on the presence of such trophic factors (Davies, A. M., *Development 100*:185-208 (1987)). Removal of such factors induces neuronal cell death, usually within 48 hrs. The death of the sympathetic neurons and sensory neurons whose survival depends on one or more members of the nerve growth factor family (nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3) can be rescued by microinjection of *bcl-2* expression vector (Garcia, I., *et al.*, *Science 258*:302-304 (1993); Allsopp *et al.*, 1993). To examine if the genes in the *Ice/ced-3* family may be responsible for neuronal cell death, the ability of *crmA* to inhibit the death of chicken dorsal root ganglionic neurons induced by NGF removal was examined. It was found that microinjection of an expression vector containing *crmA* inhibits the death of DRG neurons as effectively as that of a *bcl-2* expression vector (Gagliardini, V., *et al.*, *Science 263*:826-828 (1994)). This result demonstrated that the genes in the *Ice/ced-3* family may play a key role in regulating neuronal cell death during development.

Example 5

Results

Cloning of Ich-1

The protein product of the *C. elegans* cell death gene, *ced-3*, is homologous to the product of the mouse gene, *nedd*-2, isolated by Kumar

et al. as part of a group of genes that are down regulated during late mouse brain development (Kumar *et al., Biochem. Biophys. Res. Commun. 185:*1155-1161 (1992); Yuan, J., *et al., Cell 75*:641-752 (1993)). The *nedd*-2 cDNA in the data bank has an open reading frame of 171 amino acids and has long 3′ and 5′ untranslated regions. This 171-amino acid *nedd-2* protein does not contain the active domain, QACRG, of *ICE* and *ced-3* proteins and is homologous only to the P10 subunit of mammalian interleukin-1β converting enzyme (*ICE*) and the C-terminal part of the *ced-3* protein. While analyzing *nedd*-2 cDNA, the inventors discovered that it contains the sequence that can potentially encode a QACRG pentapeptide, but that the sequence is in another reading frame. The inventors considered the possibility that the *nedd-2* cDNA isolated by Kumar *et al.* contains cloning artifacts and that another *nedd-2* transcript could encode a protein homologous to both the P20 and P10 subunits of *ICE*.

A mouse *nedd*-2 probe was made by polymerase chain reaction (PCR). Using this mouse *nedd-2* probe, three cDNA libraries were screened: a mouse embryonic day 11.5 cDNA library from CLONTECH (one million clones screened), a human fetal brain cDNA library from James Gusella's laboratory (10 million clones screened) and a human fetal brain cDNA library from Stratagene (one million clones screened). The longest positive cDNA clones were obtained from the Stratagene cDNA library. From the Stratagene library, two cDNA species (pBSH37 and pBSH30) were identified that encode two closely related proteins homologous to the mouse *nedd-2* protein. The insert of pBSH37 (2.5 kb) encodes a protein that contains amino acid sequence similarities to both the P20 and P10 subunits of *ICE* and entire *ced-3* protein. The insert of pBSH30 (2.2 kb) contains a 61 bp additional sequence one basepair after the sequence encoding QACRG which causes an early termination of protein translation. The Northern blot analysis showed that expression patterns of this human gene are different from the expression of *nedd-2* reported by Kumar *et al.* (see below); thus, the sequences were renamed *Ich-1*$_L$ (pBSH37) (Figure 12A) and *Ich-1*$_S$ (pBSH30) (Figure 12B).

Ich-1$_S$ cDNA differs from *Ich-1*$_L$ at two locations. The first difference is at the beginning of the coding region. The putative first methionine of *Ich-1*$_S$ is 15 amino acids downstream from the first methionine of *Ich-1*$_L$ because the beginning 35 bp of *Ich-1*$_S$ is different from *Ich-1*$_L$ and includes a stop codon (Figure 12B). PCR analysis using primers specific to the first 35 bp of *Ich-1* and the *Ich-1*$_S$-specific intron (see below) and human placenta cDNA as template amplified a DNA fragment of predicted size, suggesting that the 35 bp *Ich-1*$_S$-specific sequence is not a cloning artifact and is present in the endogenous *Ich-1*$_S$ mRNA (data not shown).

The second difference is after the active domain QACRG. *Ich-1*$_S$ begins to differ from *Ich-1*$_L$ one basepair after the coding region of the active site QACRG. The difference is caused by an insertion of 61 bp sequence, which results in a termination codon 21 amino acids downstream from the insertion. The last two identical basepairs of *Ich-1*$_S$ and *Ich-1*$_L$ are AG, the general eukaryotic splicing donor consensus sequence (Mount, 1982).

Mouse genomic DNA of *Ich-1* was cloned. Analysis of mouse genomic *Ich-1* DNA showed that the 61 bp is from an intron, whose sequence is identical between human and mouse *Ich-1*. This difference between *Ich-1*$_S$ and *Ich-1*$_L$ is caused by alternative use of two different 5′ splicing donor sequences. A schematic diagram of *Ich-1*$_L$ and *Ich-1*$_S$ is shown in Fig. 13. As the result of an insertion of an intron between coding regions, the open reading frame of *Ich-1*$_S$ is broken into two: the first one encodes a 312 amino acid peptide homologous to the P20 subunit of *ICE* only and the second encodes a 235 amino acid peptide homologous to a part of the P20 subunit and the P10 subunit of *ICE*. The second is almost identical to the mouse *nedd-2* protein (Figures 12 and 13). The data suggest that only the first reading frame is translated in cells (see below).

Ich-1$_L$ protein contains similarities to both *ICE* (27% identity and 52% similarity) and *ced-3* (28% identity and 52% similarity) (Figure 14). Thus, the homology between *Ich-1* and *ced-3*, *Ich-1* and *ICE* is about equal.

Ich-1 is expressed in many tissues and THP-1 cells which express interleukin-1β converting enzyme

To characterize the function of *Ich-1*, the expression pattern of *Ich-1* was examined. Northern blot analysis of human fetal heart, brain, lung, liver and kidney tissue using the insert of pBSH37 as a probe hybridizing to both *Ich-1*$_S$ and *Ich-1*$_L$ transcripts, revealed that 4 kb *Ich-1* mRNA is expressed at low level in about same amount in all tissues examined. When the same Northern blot was analyzed using *Ich-1*$_S$ 61bp intron as a probe (which hybridizes to *Ich-1*$_S$ transcript only), it showed that *Ich-1*$_S$ was expressed in a larger amount in the embryonic heart and brain than in the lung, liver and kidney. This result suggests that in the embryonic lung, liver and kidney, *Ich-1*$_L$ is expressed in a larger amount than *Ich-1*$_S$. In Northern blot analysis of adult RNA with pBSH37 probe, *Ich-1* is detected in all the tissues examined: its level is higher in placenta, lung, kidney, pancreas than in heart, brain, liver and skeletal muscle.

To examine whether *Ich-1* and *ICE* are expressed in the same cells, a Northern blot of THP-1 cells was analyzed. *Ice* expression has been detected in these cells (Thornberry, N. A., *et al.*, *Nature 356*:768-774 (1992); Cerretti, D. P., *et al.*, *Science 256*:97-100 (1992)). The inventors found that *Ich-1* can be detected in THP-1 cells. Thus, *Ich-1* and *ICE* are both expressed in THP-1 cells.

Using quantitative RT-PCR, we examined the expression of *ICE* and *Ich-1* in the normal living T-cell hybridoma DO11.10 cells (Haskins, K., *et al.*, *Exp. Med. 157*:1149-1169 (1983)) as well as dying DO11.10 cells in serum-deprived condition. Similar to THP-1 cells, the expression of both *ICE* and *Ich-1* can be detected in DO11.10 cells. Interestingly, the expression levels of both *Ich-1*$_L$ and *ICE* appear to increase in dying DO11.10 cells under serum-deprived condition.

Overexpression of Ich-1_L induces rat-1 fibroblast death

To examine the function of *Ich-1_L*, the same transient expression system used for *ICE* (Miura, M., *et al.*, *Cell* 75:653-660 (1993)) was used to determine if overexpression of *Ich-1* induces programmed cell death. The human *Ich-1_L* cDNA was fused with the *Escherichia coli lacZ* gene and the fused gene was placed under the control of the chicken β-actin promotor (pβactH37Z). This fusion gene was transfected into Rat-1 cells by lipofectamine mediated gene transfer and the expression of the gene was examined using the X-gal reaction. Results showed that most of the blue (X-Gal-positive) Rat-1 cells transfected with pβactH37Z were round. These results are similar to those obtained with cells transfected with *mIce-lacZ* fusion sequence (Table 1). In contrast, most blue cells transfected with vector alone were flat and healthy. Live Rat-1 cells are flat while dying Rat-1 cells are round and eventually detached from plates. This result suggests that the expression of *Ich-1_L* induces Rat-1 cells to die.

To examine whether the cell death induced by *Ich-1* has any cell type specificity and to compare its effect with that of *ICE*, *mIce-lacZ* and *Ich-1-lacZ* fusion constructs were transfected to HeLa cells, NG108-15 cells, and COS cells. The cell killing effect was assayed as before (Table 1). The results showed that compared to controls, the cytotoxic effect of *Ich-1* and *ICE* exhibit certain cell type specificities. Expression of either *Ich-1* or *ICE* kill Rat-1 cells and HeLa cells effectively (>90% dead). NG108 cells are more resistant to *Ich-1* and *ICE* expression than Rat-1 cells and HeLa cells (68-80% dead). Expression of either *Ich-1* or *ICE* cannot kill COS cells (Table 1).

To examine the nuclear morphology of the cell death induced by *Ich-1* expression, the *Ich-1_L-lacZ* Rat-1 cell transfectants were stained with a rhodamine-coupled anti-β-galactosidase antibody and Hoechst dye. Results showed that β-galactosidase-positive round cells have condensed and fragmented nuclei. This is one of the characteristics of cells undergoing

apoptosis. Thus, the results suggest that overexpression of *Ich-1*$_L$, like that of *ICE*, causes Rat-1 cells to undergo programmed cell death.

To determine if cell death caused by overexpression of *Ich-1*$_L$ is specific, three mutant *Ich-1*$_L$ fusion proteins were made: the first was a Ser → Cys 303 in the active site of *Ich-1*, the second was a Thr → Ala 352 in the putative P10 subunit and the third with a Phe → Leu 212 in the putative P20 subunit (Fig. 14). The Ala 352 in P10 and Leu 212 in P20 are two amino acid residues of *ced-3* that are conserved in *Ich-1* but not in *ICE*. The mutant *Ich-1*$_L$*-lacZ* fusion constructs were transfected into Rat-1 cells and the expression was examined by X-gal reaction as before.

The analysis revealed that the S303C and T352A mutations eliminated the activity of *Ich-1* completely (Table 1) while F212L mutation caused a reduction of cell killing activity of *Ich-1*$_L$ (Table 1). These results suggest that the ability of *Ich-1* to cause cell death depends upon its enzymatic activity and that only some characteristics of *ced-3* are conserved in *Ich-1*.

The cell death induced by overexpression of *Ice* can be inhibited by *bcl-2* and *crmA* (Miura, M., *et al.*, *Cell 75*:653-660 (1993)). To examine if the cell death induced by expression of *Ich-1* could also be inhibited by *bcl-2* and *crmA*, *Ich-1*$_L$*-lacZ* fusion construct was transfected into Rat-1 cells that overexpress either *bcl-2* or *crmA* (Miura, M., *et al.*, *Cell 75*:653-660 (1993)). Cell death was assayed as described for Table 1. The results showed that the cell death induced by overexpression of *Ich-1* could be inhibited effectively by *bcl-2* but only marginally by *crmA*.

Expression of Ich-1$_S$ protects Rat-1 fibroblast death

Since *Ich-1*$_S$ contains two open reading frames, it was important to determine which reading frame is functionally translated. *Ich-1*$_S$ was translated in the presence of ^{35}S-methionine using *in vitro* transcribed RNA in a reticulocyte lysate as described in Experimental Procedures. The translated products were run on an SDS-polyacrylamide gel with molecular weight

standards. *Ich-1$_S$* antisense RNA was used as a negative control. Results showed that only the first reading frame was translated.

Second, *E. coli lacZ* gene was fused to the ends of first (pβactH30Z1) and second (pβactH30Z2) open reading frames. The constructs were separately transfected into Rat-1 cells and the cells were assayed for color using the X-gal reaction. Results showed that only when *LacZ* gene was fused to the end of the first open reading frame (but not the second open reading frame) could blue cells be detected. Thus, it is most likely that only the first open reading frame of *Ich-1$_S$* homolog is used *in vivo*.

To characterize the function of *Ich-1$_S$*, the ability of pβactH30Z1 to cause cell death was examined. pβactH30Z1 was transfected in Rat-1 cells and the X-gal reaction was developed as before. The analysis showed that the expression of pβactH30Z1 did not cause cell death (Table 1).

To examine if *Ich-1$_S$* has any protective effect against cell death, a stable Rat-1 cell line that express *Ich-1$_S$* was established. The cDNA *Ich-1$_S$* was cloned into pBabepuro retroviral expression vector (Morgenstern *et al.*, *Nucl. Acids Res. 18*:3587-3596 (1990)) and transfected into Rat-1 cells. The stable transfectants were selected in puromycin and individual clones were assayed for expression of *Ich-1$_S$* by Northern blot analysis. The clones that expressed *Ich-1$_S$* were used for analysis and the clones that did not express *Ich-1$_S$* were used as negative controls together with untransfected Rat-1 cells. When plated in non-confluent density and washed carefully, Rat-1 cells would die in serum-free medium. Under these conditions, Rat-1 cells expressing *bcl-2* or *crmA* were resistant to death (Fig. 15). When the ability of the stable Rat-1 cell lines that express human *Ich-1$_S$* was tested under serum-free conditions, it was found that they are more resistant to serum deprivation than parental Rat-1 cells and negative control transfectants not expressing *Ich-1$_S$* (Fig. 15). These experiments suggest that *Ich-1$_S$* may have the ability to prevent cell death.

Since *Ich-1$_S$* may prevent cell death by inhibiting *Ich-1$_L$*, the inventors examined whether Rat-1 cells express *Ich-1*. Using mouse *Ich-1* cDNA as a

probe, an mRNA species predictive of the *Ich-1* transcript was detected in Rat-1 cells under low stringency conditions.

Discussion

The isolation and characterization of *Ich-1*, a mammalian gene belonging to the cell death gene family of *Ice/ced-3*, has been described. Two distinct *Ich-1* mRNA species have been identified (*Ich-1*$_L$ and *Ich-1*$_S$). These two cDNAs differ in both 5′ regions around translation initiation and in the middle region. The difference in the middle is the result of alternative use of two different 5′ splicing donor sites.

The *Ich-1* gene is expressed at low levels in both embryonic and adult tissues tested. *Ich-1*$_S$ is expressed at higher levels than *Ich-1*$_L$ in embryonic heart and brain. The converse is true in embryonic lung, liver and kidney. The expression of both *ICE* and *Ich-1* can be detected in THP-1 cells and DO11.10 cells. The expression of both *ICE* and *Ich-1*$_L$ appear to increase in dying cells under serum deprived conditions. Overexpression of *Ich-1*$_L$ in rat fibroblast cells caused programmed cell death. This suggests that *Ich-1* is also a programmed cell death gene. Overexpression of *Ich-1*$_S$ did not cause cell death. Stable expression of *Ich-1*$_S$ prevented Rat-1 cell death induced by serum deprivation. The collective results show that *Ich-1* encodes protein products that regulate cell death positively and negatively.

The mouse *nedd*-2 gene was originally isolated by Kumar *et al.* (*Biochem. & Biophy. Res. Comm. 185*:1155-1161 (1992)). The *nedd*-2 gene was identified as having a transcript of 3.7 kb that is abundantly expressed in embryonic day 10 mouse brain and almost undetectable in adult brain. The *nedd*-2 cDNA isolated contained an open reading frame of 171 amino acids and long 5′ and 3′ untranslated regions with stop codons in all reading frames. The 171-amino-acid open reading frame is homologous to P10 subunit of *ICE* and the C-terminal part of *ced-3* protein (Yuan, J., *et al.*, *Cell 75*:641-752 (1993)).

In the Northern blot analysis described herein, the *Ich-1* expression in human fetal brain is not high compared to other tissues tested (heart, lung, liver and kidney). Part of the difference could be explained by the different developmental stages tested: mouse E10 versus human 20-26 week old fetuses. However, *Ich-1* expression can be detected in human adult tissues.

In the studies herein, amplification of the 5' untranslated regions of the mouse *nedd*-2 cDNA that Kumar reported was not achieved. It is possible that the 5' untranslated region in the Kumar clone was a product of incompletely processed *nedd*-2 mRNA. Both *Ich-1* mRNAs are about 4 kb; since the cDNA clones described herein are 2.5 kb and 2.2 kb for $Ich\text{-}1_L$ and $Ich\text{-}1_S$, respectively, these cDNAs are incomplete. However, since they are fully functional in the assay reported herein, the complete coding regions should be encoded in these two cDNAs.

Ich-1 is a new member of the *ICE/ced-3* family of cell death genes. Thus, unlike *C. elegans*, mammals must have multiple members of *ICE/ced-3*. *Ich-1* is even slightly more homologous to *ced-3* protein than *ICE*. The cell death induced by overexpression of *Ich-1* was poorly inhibited by *crmA*. This result is similar to that with *ced-3* (Miura, M., *et al.*, *Cell* 75:653-660 (1993)).

The two amino acid residues of *ced-3* protein that are conserved in *Ich-1* but not in *ICE* were mutagenized. Results showed that T352A completely eliminated the ability of *Ich-1* to cause cell death, despite the fact that the corresponding amino acid in *ICE* is a Ser, while F212L caused a reduction of the cell killing activity. These data also suggest that *Ich-1* may be mechanistically more similar to *ced-3* than *ICE*, and *Ich-1* and *ICE* may have evolved independently from *ced-3*.

The overexpression of *ICE* and *Ich-1* can kill Rat-1 cells and HeLa cells effectively but NG108 cells only moderately. The possibility that the activity of β-actin promoter is lower in NG108 cells cannot be ruled out. However, an interesting possibility is that NG108 cells express a higher level of *ICE* and *Ich-1* inhibitors. COS cells are completely resistant to the cell killing activity of *ICE* and *Ich-1*. COS cells may lack either the activator or

the substrates of *ICE* and *Ich-1*. This result also suggests that the cytotoxic effects of *ICE* and *Ich-1* have certain specificity and are unlikely to be caused by random cleavage activities of proteases.

Ich-1 can make a protein product that either prevents or causes cell death depending on how the mRNA is processed. Similar regulation has been observed with *bcl-x*, a *bcl-2* related gene (Boise *et al.*, 1993). The *bcl-x* transcripts can also be processed in two different ways: the larger mRNA, *bcl-x*$_L$, encodes a *bcl-2* related protein product that can inhibit cell death induced by growth factor withdrawal when overexpressed in an IL-3-dependent cell line. Alternative splicing of *bcl-x* transcripts can generate another smaller transcript. *bcl-x*$_S$, encodes an internal truncated version of *bcl-x* protein that inhibits the ability of *bcl-2* to enhance the survival of growth factor-deprived cells. Control of the RNA splicing could prove to be an important differential regulatory check point in programmed cell death.

How does *Ich-1*$_S$ act to prevent cell death? It could act either by inactivating the activator of cell death or by directly inactivating *Ich-1*$_L$. Since Rat-1 cells appear to express *Ich-1*, these two possibilities cannot be distinguished at present. In the transient transfection assay, the expression of *Ich-1*$_L$-lacZ fusion gene and the *Ice*-lacZ fusion gene kill the stable *Ich-1*$_S$ expressing cells as efficiently as the control Rat-1 cells (L. Wang, unpublished data). Thus, unlike *crmA* or *bcl-2*, the inhibition of cell death by *Ich-1*$_S$ may be highly dosage-dependent. This is probably why the expression of *Ich-1*$_S$ provided only partial protection to the cell death of Rat-1 cells induced by serum deprivation: only those cells expressing high levels of *Ich-1*$_S$ are protected.

crmA has the ability to suppress cell death induced by overexpression of *Ich-1*$_L$. The amino acid sequence of *crmA* protein is homologous to the members of the serpin and superfamily (Pickup *et al.*, 1986), which usually inhibit serine proteases by acting as pseudosubstrates. The nature of interaction of *ICE* and *crmA* protein is not fully understood but it is likely to be similar to the interaction of other serpin and serine proteases. The

inhibition of *ICE* family members by *crmA* may depend upon both the affinity and relative concentration of *ICE*s and *crmA* protein. The fact that *crmA* can suppress a certain percentage of cell deaths induced by overexpression of the *Ich-1*$_L$ suggests that *crmA* and *Ich-1* can bind to each other although their affinity may be low. It is possible that when *Ich-1* concentration is lower, *crmA* may be able to suppress a much larger percentage of cell death induced by *Ich-1*. Microinjection of *crmA* expression construct can effectively suppress the death of dorsal root ganglia neurons induced by nerve growth factor deprivation (Gagliardini, V., *et al.*, *Science 263*:826-828 (1994)). One or more *ICE/ced-3* family members may be responsible for neuronal cell death. When *crmA* expression construct is microinjected into neurons, the transient concentration of *crmA* protein may be very high. Thus, it is possible that *crmA* may be able to suppress multiple members of *ICE/ced-3* family under such conditions despite the fact that their affinity to *crmA* is not very high.

Since the expression of *Ich-1* and *Ice* can be detected in the same cells, the results described herein suggest that multiple members of *Ice/ced-3* family may contribute to cell death induced by a single signal. There are three possible ways that *Ice* and *Ich-1* may act to cause cell death (Figure 16). First, *Ich-1* may activate Ice, directly or indirectly, to cause cell death. Second, *ICE* may inactivate *Ich-1*, directly or indirectly, to cause cell death. Third, *ICE* and *Ich-1* may act in parallel to cause cell death. In the first scenario, the inhibitor of *ICE* should inhibit cell death induced by *Ich-1*. In the second scenario, the inhibitor of *Ich-1* should inhibit the cell death induced by *ICE*. To test this hypothesis, specific inhibitors for each member of *ICH* are necessary. For the reasons discussed above, it seems likely that *crmA* can inhibit other members of *ICE/ced-3* family as well. These models can be tested directly by "knock-out" mutant mice in which a specific member of the *ICE/ced-3* family is mutated.

Experimental Procedures

Cloning and Construction of Plasmids

The mouse *nedd*-2 cDNA was isolated using embryonic mouse brain cDNA and the primer pairs specific for the 5′ and 3′ untranslated regions and the coding region. Primers *nedd2/1* (5′-CAACCCTGTAACTCTTGATT-3′) and *nedd2/2* (5′-ACCTCTTTGGAGCTACCAGAA-3′) were used for amplifying the 5′ untranslated region. Primers *nedd2/3* (5′-CCAGATCTATGCTAACTGTCCAAGTCTA-3′) and *nedd2/4* (5′AAGAGCTCCTCCAACAGCAGGAATAGCA-3′) were used for amplifying the *nedd-2* coding region. Primer *nedd2/5* (AGAAGCACTTGTCTCTGCTC) and *nedd2/6* (5′TTGGCACCTGATGGCAATAC-3′) were used for amplifying the 3′ untranslated region. 0.5 kb PCR product of *nedd-2* coding region was cloned into pBluescript plasmid vector to be used as a probe (Stratagene).

A human fetal brain cDNA library (Stratagene) was screened with murine *nedd*-2 cDNA probe at low stringency. The filters were hybridized in 5x SSPE, 30% formamide, 1x Denhardt's solution, 1% SDS at 42°C overnight and washed in 1x SSPE and 0.5% SDS, twice at room temperature and twice at 45°C (20 min). The human *Ich-1*$_S$ (pBSH30) was isolated from the positive clones using a *BamH*I-*Sal*I fragment of the murine *nedd*-2 cDNA, a 76 bp fragment which contains the 61 bp intron, as a probe under the same hybridization and washing conditions described above. The phage clones (pBSH37 for *Ich-1*$_L$, pBSH30 for *Ich-1*$_S$) were excised *in vivo* to obtain plasmids by an *in vivo* excision protocol (Stratagene). To construct expression constructs, PCR was performed using synthetic primers. H1 (5′-GATATCCGCACAAGGAGCTGA-3′) and H2 (5′-CTATAGGTGGGAGGGTGTCC-3′) were used for *Ich-1*$_L$ construction. H3 (5′-GATATCCAGAGGGAGGGAACGAT-3′), corresponding to sequences in the 5′ region of *Ich-1*$_S$ cDNA and H4

(5'-GATATCAGAGCAAGAGAGGCGGT-3'), corresponding to the sequences in the 3' region of the first open reading frame (ORF) of *Ich-1*$_S$ were used for the first ORF of *Ich-1*$_S$ construction. H3 and H5 (5'-GATATCGTGGGAGGGTGTCCT-3'), corresponding to the sequences in the 3' region of the second ORF of *Ich-1*$_S$ were used for the second ORF of *Ich-1*$_S$ construction. pBSH37 and pBSH30 were used as templates where appropriate. The three PCR products were inserted into the *Eco*RV site of pBluescript II, and the inserts were isolated by digestion with *Sma*I and *Kpn*I and cloned into *Sma*I-*Kpn*I sites of *BSLacZ* (Miura, M., *et al.*, *Cell 75*:653-660 (1993)). *Not*I linkers were added to the *Kpn*I site by digesting with *Kpn*I, blunt-ending by T4 polymerase and ligating in the presence of excess *Not*I linker. These constructs, BSh37Z, BSh30Z1 and BSh30Z2, were digested with *Not*I and individually cloned into pβactstneoB (which uses chicken β-actin promoter) (Miyawaki, A., *et al.*, *Neuron 5*:11-18 (1990)). The final plasmids were designated pβactH37Z, pβactH30Z1 and pβactH30Z2, respectively. pBabeH30 plasmid, used for establishing stable Rat-1 cell line carrying *Ich-1*$_S$, was constructed by inserting the full length *Ich-1*$_S$ cDNA into the *Sal*I site of pBabe/puro vector (Morgenstern, J. P., *et al.*, *Nucl. Acids Res. 18*:3587-3596 (1990)).

To mutagenize Cys 303 to a Ser residue in the active domain of *Ich-1*$_L$, Ala 352 to a Thr residue in the P10 subunit of *Ich-1*$_L$ and Leu 212 to a Phe residue in the P20 subunit of *Ich-1*$_L$, primers containing mutant sites were synthesized as follows:

HM1 5'-ATCCAGGCCTCTAGAGGAGAT-3'

HM2 5'-ATCTCCTCTAGAGGCCTGGAT-3'

HM3 5'-TGCGGCTATACGTGCCTCAAA-3'

HM4 5'-TTTGAGGCACGTATAGCCGCA-3'

HM5 5'-CACAGTACTTTCGTCACCCT-3'

HM6 5'-AGGGTGACGAAAGTACTGTG-3'

(HM1 is corresponding with HM2, HM3 is corresponding with HM4, HM5 is corresponding with HM6). PCRs were performed in two steps. To make the Cys 303 to Ser mutation, in the first round of PCR, the fragments from the N-terminal to mutation site of *Ich-1*$_L$ and from the mutant site to C-terminal of *Ich-1*$_L$ were synthesized using two primer pairs, T3 and HM1, HM2 and T7, and PBSH37 as a template. In the second round PCR, the two PCR fragments generated in the first reaction were used as templates and T7 and T3 were used as primers. Two such rounds of PCR generated a full length *Ich-1*$_L$ mutant. The other two mutations were generated in similar way using T3 and HM3, HM4 and T7 for Ala 352 to Thr mutation, and T3 and HM5, HM6 and T7 for Leu 212 to Phe mutation as primers for first PCR. The PCR products were inserted into the *Eco*RV site of pBluescript II and sequenced. The mutant cDNA inserts were cloned into expression vectors as described above. The mutated clones were designated pβactH37ZCS, pβactH37ZAT and pβactH37LF.

Cell Culture and Functional Studies

All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). The day before transfection, cells were seeded at a density of about 2.5×10^5 in each of the 6-well dishes. For each well, 0.7-1μg of *LacZ* chimeric construct and 10μg of lipofectamine reagent were used according to a protocol from GIBCO BRL (Gaithersburg, MD). The cells were incubated for 3 hr in serum-free medium

containing DNA and lipofectamine. Then an equal volume of growth medium containing 20% serum was added without removing the transfection mixture and incubation was continued for 24 hr. The expression of the chimeric gene in cells in culture was detected as previously described (Miura, M., *et al.*, *Cell* 75:653-660 (1993)).

To establish Rat-1 cell lines overexpressing *Ich-1$_S$*, pBabeH30 was transfected into Rat-1 cells using lipofectamine mediated gene transfer. Resistant cells were selected using 3μg/ml puromycin for about 10 days. Cells were assayed for expression of *Ich-1$_S$* by Northern blot analysis. To examine whether *Ich-1$_S$* can render Rat-1 cells resistant to apoptosis in the condition of serum deprivation, the Rat-1 cells overexpressing *Ich-1$_S$*, untransfected control Rat-1 cells, transfected negative control Rat-1 cells and Rat-1 cells overexpressing *bcl-2* or *crmA* were seeded in 24-well dish at 5x10^4 cells in 500 μl of DMEM containing 10% FCS for 24 hr, and then washed once with serum-free DMEM and transferred into 500 μl of serum-free DMEM. The cells were harvested at daily intervals and stained with 0.4% trypan blue for 5 min. at room temperature. The numbers of dead and living cells were counted using a haemocytometer.

RNA Analysis

The Multiple Tissue Northern (MTN) blots membrane of human fetal and adult tissues (CLONTECH) were probed using human *Ich-1$_L$* cDNA or the intron of *Ich-1$_S$* cDNA (for fetal tissue) under conditions of 5x SSPE, 10x Denhardt's solution, 50% formamide, 2% SDS, 100μg/ml salmon sperm DNA at 42°c for overnight. The blots were washed twice in 2x SSPE, 0.05% SDS at room temperature and twice in 0.1x SSPE, 0.1% SDS for 20 min. at 50°c for 20 min each.

In Vitro Transcription and Translation of Ice-ced 3_S homolog

To determine which open reading frame of *Ich-1_S* homolog was expressed, pBluescript plasmid containing *Ich-1_S* (pBSH30) was linearized at the 3′ multiple cloning site with *Xho*I, purified, and transcribed with T3 RNA polymerase for 2 hr at 37°c using a protocol from Stratagene. The plasmid was also linearized at the 5′ multiple cloning site with *Not*I, purified, and transcribed with T7 polymerase as an antisense control. The resulting runoff transcripts were extracted with phenol-chloroform and ethanol precipitated. *In vitro* translation was performed with rabbit reticulocyte lysate (Promega) in the presence of ^{35}S-methionine for 1 hr. at 30°C. 5μl lysate was mixed with equal volume of 2xSDS gel loading buffer and subjected to SDS-polyacrylamide gel electrophoresis (12%). The gel was dried and exposed to X-ray film.

TABLE 1

Expression cassettes	COS	HeLa	NG108-15	Rat-1	Rat-1/bcl-2	Rat-1/*crmA*
pactbβgal'	• 1.3+0.1(983)	2.9±0.2(1020)	4.2±0.2(1535)	2.9±0.2(1470)	3.4±0.2(1446)	3.7±0.1(1459)
pβactM10Z	• 11.0±0.2(1080)	93.9±0.3(1003)	80.2±0.5(1545)	94.2±1.1(978)	28.8±0.5(691)	45.8±1.6(233)
pβactH37Z	8.3±0.9(1053)	91.4±0.2(1076)	68.7±1.5(1605)	92.1±0.3(1079)	21.5±3.2(1335)	80.7±0.9(1010)
pβactH37ZCS	ND	5.6±0.1(1039)	5.9±0.9(707)	4.1±0.2(1477)	ND	ND
pβactH37ZAT	ND	8.2±0.7(435)	5.2±0.2(640)	5.4±0.3(1356)	ND	ND
pβactH37ZLF	ND	75.8±2.2(1404)	39.9±3.8(1193)	77.4±0.4(1704)	ND	ND
pβactH30Z1	1.3±0.2(676)	0.0±0.0(40)	0.0±0.0(61)	1.8±0.4(785)	ND	ND

Table 1. The constructs as described in the text were transiently transfected into Rat-1 cells, Rat-1 cells expressing human *bcl-2*, Rat-1 cells expressing cowpox virus *crmA* gene, HeLa cells, NG108-15 cells and COS cells. Cells were fixed lightly 24 hr after transfection and stained with X-Gal for 3 hr. The data (mean±SEM) shown are the percentage of round blue cells among total number of blue cells counted. The numbers in the parentheses are the number of blue cells counted. The data were collected from at least three independent experiments. ND = not determined.

What Is Claimed Is:

1. A method of preventing programmed cell death in vertebrates comprising the step of inhibiting the enzymatic activity of interleukin-1β converting enzyme (*ICE*).

2. The method of claim 1, wherein said enzymatic activity is inhibited by an *ICE*-specific antiprotease.

3. The method of claim 2, wherein said antiprotease is *crmA*.

4. A method of promoting programmed death in vertebrate cells by increasing the enzymatic activity of *ICE* in said cells.

5. The method of claim 4, wherein said vertebrate cells are cancer cells.

6. The method of claim 5, wherein said cancer cells overexpress the oncogene *bcl-2*.

7. A substantially pure gene which is preferentially expressed in thymus and placental cells and which encodes a protein causing programmed cell death.

8. The gene of claim 7, wherein said protein has the amino acid sequence shown in Figure 6.

9. The gene of claim 8, wherein said gene has the cDNA sequence shown in Figure 6.

10. An expression vector having the gene of either claim 8 or claim 9.

11. A host cell transformed with the vector of claim 10.

12. A substantially pure protein wherein said protein is preferentially expressed in thymus or placental cells and which causes the death of said cells.

13. The protein of claim 12, wherein said protein has the amino acid sequence of *mICE2* as shown in Figure 6.

14. A functional derivative of the protein of claim 13.

15. A method of promoting programmed cell death in thymus or placental cells comprising the step of increasing the activity of the protein of claim 7.

16. A substantially pure DNA molecule comprising the cDNA sequence of human *Ich-1* as shown in Figure 8.

17. An expression vector having the DNA of claim 16.

18. A host cell transformed with the vector of claim 17.

19. A substantially pure protein comprising the amino acid sequence of human *Ich-1* as shown in Figure 8.

20. A functional derivative of the protein of claim 19.

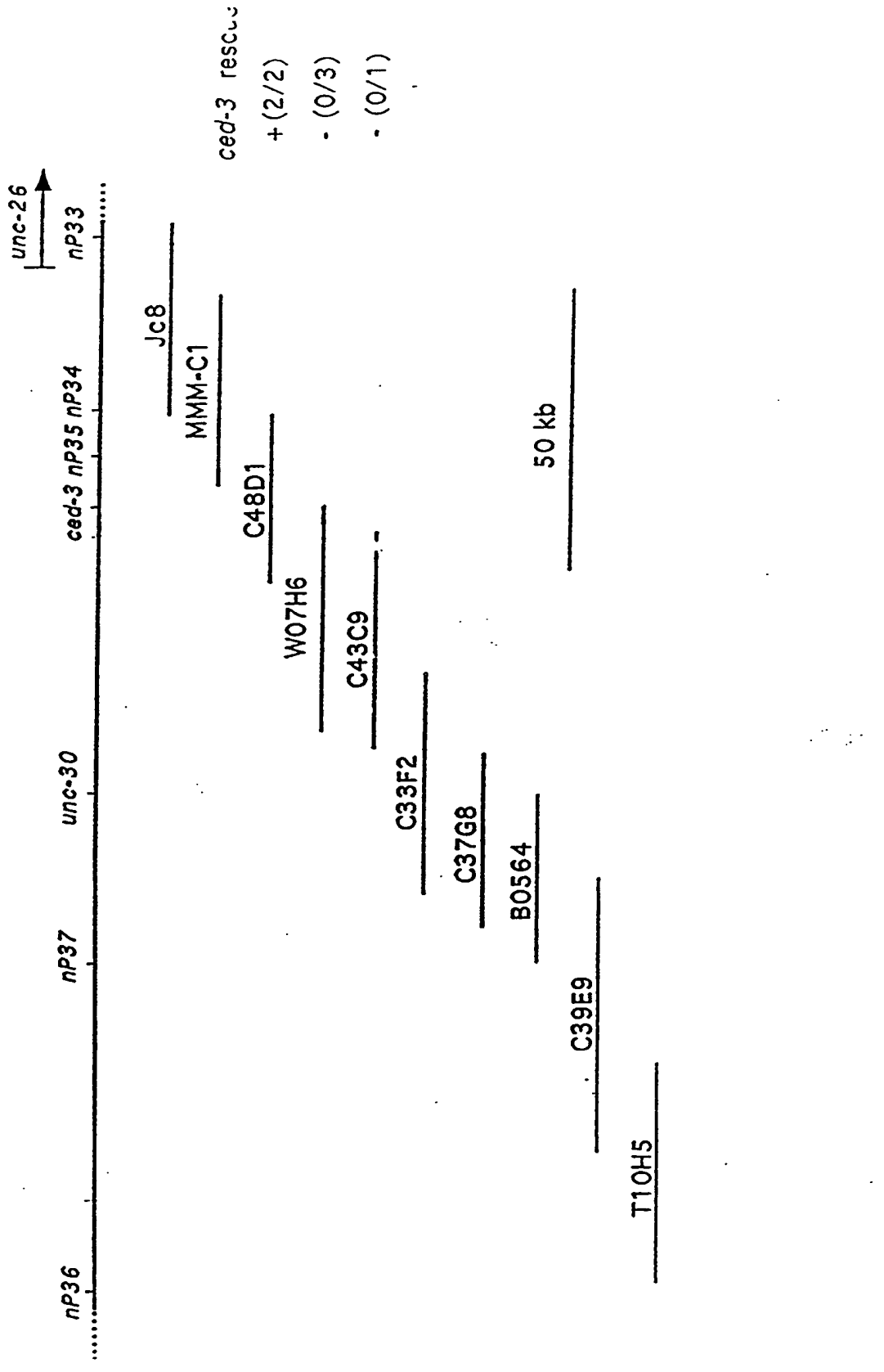
21. A substantially pure DNA molecule comprising the cDNA sequence of human *Ice-4* homolog as shown in Figure 16.

22. An expression vector having the DNA of claim 21.

23. A host cell transformed with the vector of claim 22.

24. A substantially pure protein comprising the amino acid sequence of human *Ice-4* homolog as shown in Figure 16.

25. A functional derivative of the protein of claim 24.


unc-26
nP33
ced-3 rescue
+ (2/2)
- (0/3)
- (0/1)
Jc8
MMM-C1
C48D1
W07H6
C43C9
C33F2
C37G8
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T10H5
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nP36
nP37
unc-30
ced-3 nP35 nP34

FIGURE 1

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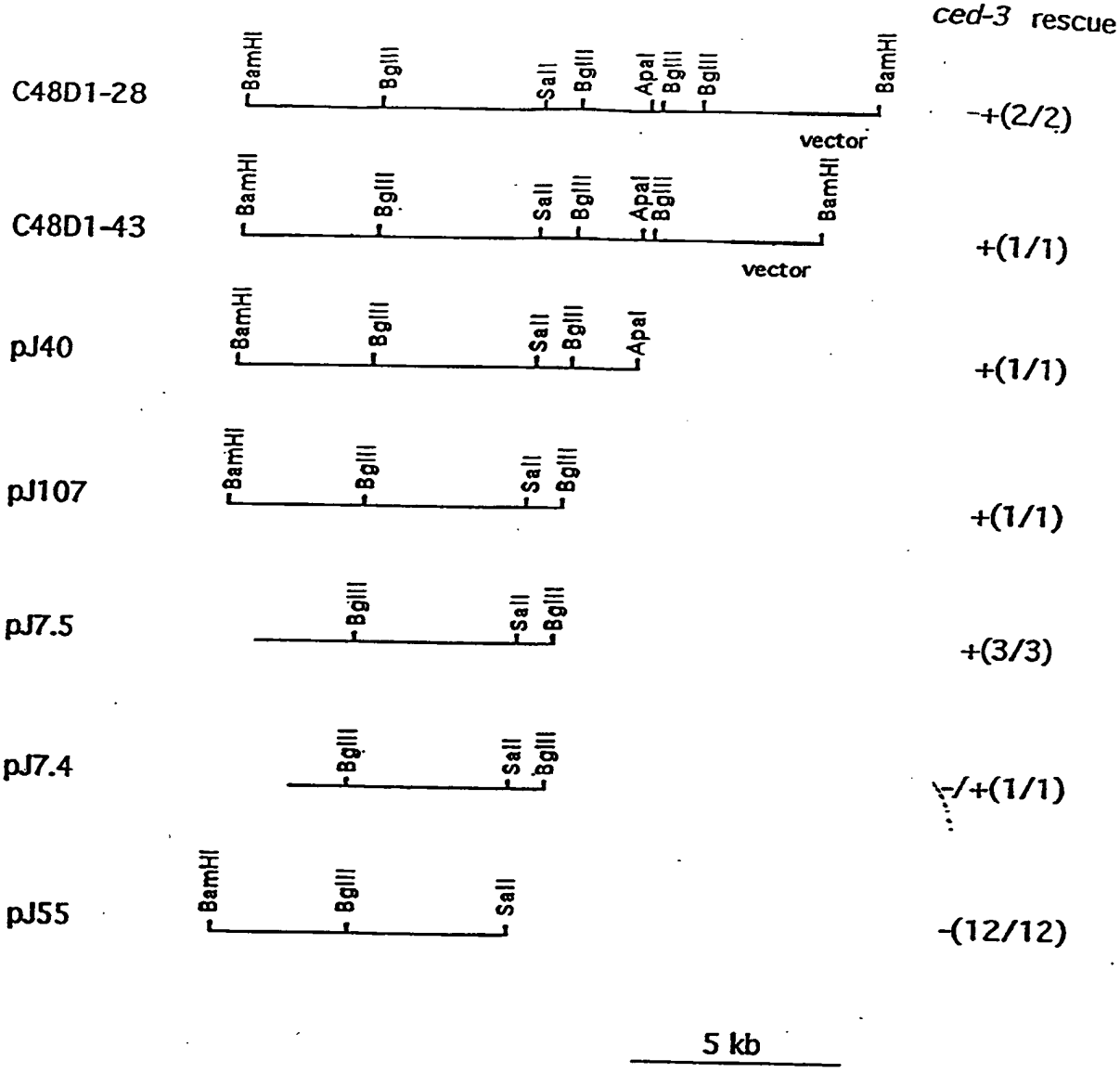

FIGURE 1A

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3a

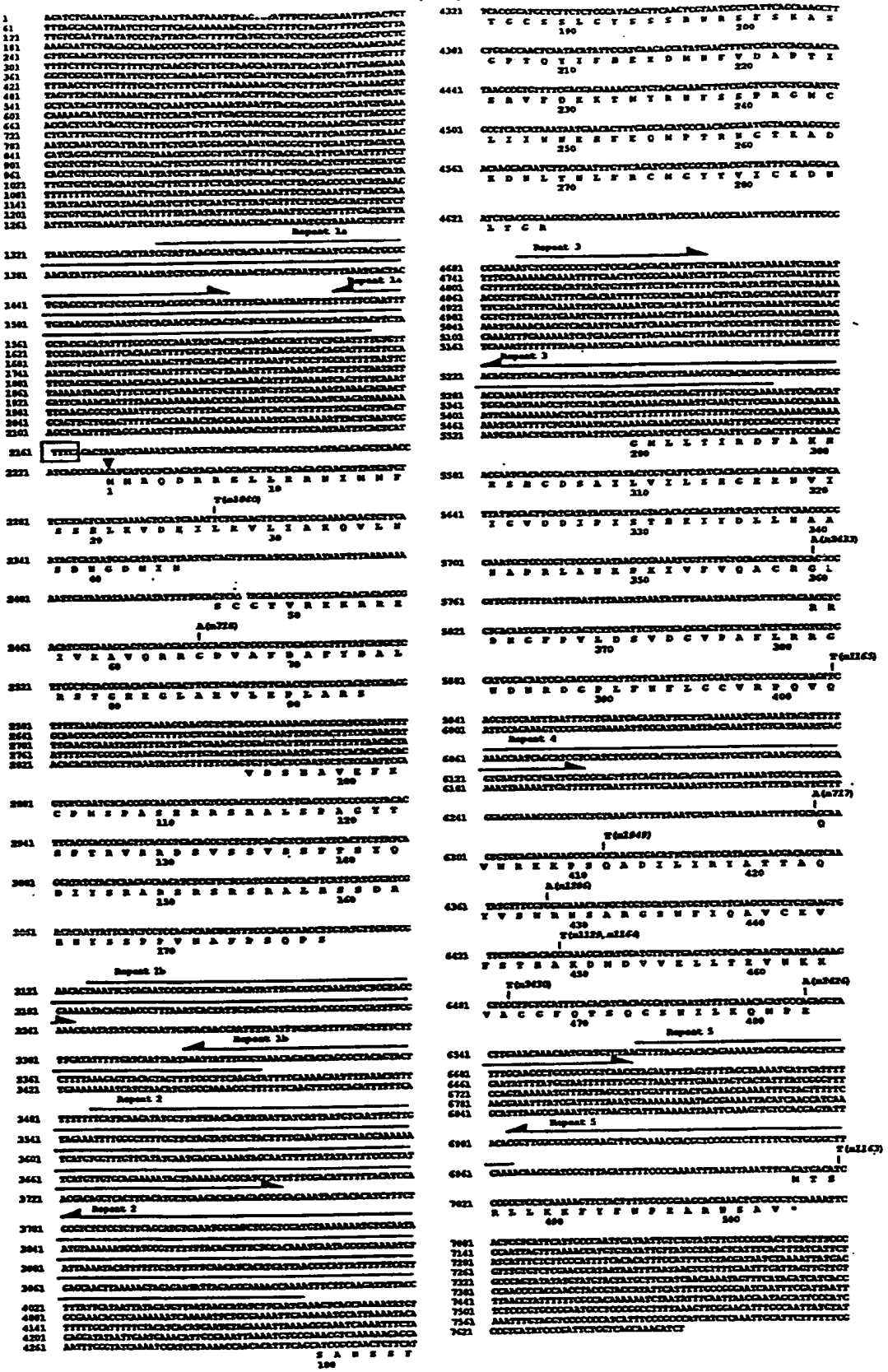

FIGURE 2

Repeat 1

```
                    1       10        20        30        40        50        60        70
ced-3(1a,for)       GTATTAA-GGAATCACAAAATTCTGAGAATGCGTACTGCGCAACATATTTGACGG-CAAATATCTCGTAGCG
ced-3(1a,rev)       ..................AAAATTCAGAGAATGCGTATTACAGTC-ATATTTGGCGCGCAAAATATCTCGTAGCT
ced-3(1b,for)       ..................AAATTCTGAGAATGCGCATTACTCAACATATTTGACGCGC-AAATATCTCGTAGCG
ced-3(1b,rev)       ..........................................................TATCTTGAAGCG
fem-1(for)          GTATTAC-GGCAAGAAATAATTATGAGAATGCCTATTGCGCACCATAGTTGACGCGCAAAATATCTCGTAGCG
fem-1(rev)          GTATAAC-GGTAACACACAATTCTGAGAATGCGTATTGCACAACACATTTGACGCGCAAAATATCTCGTAGCG
hlh-1(for)          CTATTAC-GGGAGTACAAAATTCTGAGAATGCGTACTGCGCAACATATTTGACGCGCAAAATATTTCGTATCG
hlh-1(rev)          .........GGGAGCACAAAATTCTGACTATGAGAAT-GCGTATAA-.........GCACAAAATATTTCGTAGCG

                    1       10        20        30        40        50        60        70

consensus           -TAT-A--GG-A--A-A-AATT--GA--ATG---A-T-C-----A-A-TTG-CG--CAAAATAT-T-G-A-C-

                    80      90        100       110       120       130       140

ced-3(1a,for)       AAAACTACAGTAATTCTTTAAATGACTACTGTAGCGC-----TTGTGTCGA-TTTACGGGCTCAATT---------
ced-3(1a,rev)       AGAACTACAGTAATCCTTTAAATGACTACTGTAGCG-------TTGTGACGA-TTTACGGGTTATCAAAATTCGAAA
ced-3(1b,for)       AAAA-TACAGTAACCCTTTAAATGACTATTGTAG---------TGTCGA-TTTACGGGC--TCGATTTTCGAAA
ced-3(2b,rev)       AAAACTACTGTAACTCTTTAAAAGAGTACTGTAGCGC-----TGGTGTCTG-TTTACGGAAATAATT---------
fem-1(for)          AAAACTACAGTAACTCTTTGAATGACTACTGTAGCGC-----TTGTTTCGA-TTTACGGGCTCGTT----------
fem-1(rev)          AAAACTACAGTGATTCGCTGAATGAATACGGTAGGGTCG---------------------------------------
hlh-1(for)          AAAACTACAGTAATTCGTTTATTGGCTACTGT-GCG---------TGTTGA-TTTACGGGC----------------
hlh-1(rev)          AAAACTACAGTAATTTGTCAAGGGACTACTGTAGCTAGCG-CTTGTGTCGA-TTTACGGAGC-TCGATTTT-----

                    A-AACTAC-GT-A--------A--G--TA--GTAG---------T-GT-------TTTACGG-----------TT-GAAA

consensus
```

FIGURE 2A(i)

Repeat 2

```
                1      10        20        30        40        50        60        70        80
ced-3(for)      TCATTCAAGATATGCTTATTAACACATATAATTATCATTAATGTGAATTTCTTGTAGAAATTTTGGGCTTTTCGTTCTAG
ced-3(rev)      TCATTCAAGATATGCTTATTAACATCTATAATTATCAATAAAGGTAATATCTTGAAGAAATTTTGG--TTTTCGCTCTAA

consensus       TCATTCAAGATATGCTTATTAACA--TATAATTATCA-TAA-G--AAT-TCTTG-AGAAATTTTGG--TTTTCG-TCTA-

                   90       100       110       120       130       140       150       160
ced-3(for)      TATGCTCTACTTTTGAAATTGCTCAACGAAAAAAT----------CATGTGTTTGTTCATATGAATGACGAAAAATA
ced-3(rev)      TATTCTCTACTTTTTAAGTTGCTCAACGAAAAAAATAATGGGGTTAATCATGTG----------ATGTTGAAAAATA

consensus       TAT-CTCTACTTTT-AA-TTGCTCAACGAAAAAAT----------CATGTG----------ATG--GAAAAATA

                  170       180       190       200       210       220       230       240
ced-3(for)      GCAA-----T-TTTTTATATATTTT-CCCCTATTCATGTTGTGCAGAAAAATAGTAAAAAAGCGCATGCATTTTT-----
ced-3(rev)      CAAAAAATGTATTTTAATACATTTTCCCCCTATTCAT-TTGTGCAGAAAAGT-GTAAAAAAACGCATGCATTTTTTACAT

consensus       --AA-----T-TTTT-ATA-ATTTT-CCCCTATTCAT-TTGTGCAGAAAA-T-GTAAAAAA-CGCATGCATTTTT-----

                  250       260       270       280       290
ced-3(for)      ----CGACA-TTTTTTACATCGAACGACAGCTCACTTCACATGCTGAAGACGAGAGACG
ced-3(rev)      TATTCGACATTTTTTTTACATCGACCGAGATCCCCATTTCACATGCTGAAGACGAGAGACG

consensus       ----CGACA-TTTTTTACATCGA-CGA-A-C-CA-TTCACATGCTGAAGACGAGAGACG
```

FIGURE 2A(ii)

Repeat 3

```
                                1        10        20        30        40        50        60
ced-3(for)                      CAGCTTCGAGAGTTTG-AAATTACAGTACTCCTTAAAGGCGCACACCCCATTTGCATTGG
ced-3(rev)                      ..........................................................
lin-12(for)                     CAGCAACAAATGTTTG-AAATTACAGTAATCTTTAAAGGCGCACACC-..........
lin-12(rev)                     ..........................................................
BO303(1)                        CTGCAACGAAAGTCTG-AAATTACAGTACCCCTTAAAGGCGCATA-............
BO303(2)                        ..........-GTTAG-AAACTAGAGTACCTCTTAAAGGCGCACAT--CCTTTCCCACCT
ZK643(1)                        CAGCAACAAAAGTTTG-AAATTACAGTGCTCTTTAAAGGCACACACC-TTTTTACATT-T
ZK643(2,for)                    CAGAAGCGAAAATTTG-AAATTACAGTACTCTTTAAACGCTCAA-CCCCGTTTCTATTCA
ZK643(2,rev)                    .......-GATTGTT-GAAAATTACAGTAATCTTTAAAGGCGCACACA-CGTTTGTATTTT
ZK643(3)                        ..........................................................
glp-1(for)                      ..........TTTTTAAACTACAGTACTCTTTAGGAGCGCACA--TTTTTTCGCATTT
glp-1(rev)                      ........ACCGTTTG-AAACTACAGTACTCTTTAAAGGCGC-..............
consensus                       C-G---C-----T----AAA-TA-AGT-----TTA---GC-CA-A-----TTT-.....

                                    70        80        90       100       110
ced-3(for)                      ACCAAAAATTTGTCGTGTCGAGA-CCAGGTA-CCGTAGTTTTTG-TC------GCAAA
ced-3(rev)                      ---ACAAA-TTGTCGTGTCGAGA-CCGGGCG-CCACA-....................
lin-12(for)                     ..........................................................
lin-12(rev)                     AACAAAACTTTGTCGTGTCGAGA-CCGGGTA-CCGTATTTTTAATT--------GCAAA
BO303(1)                        ..........................................................
BO303(2)                        ATCGAAAATTTGTCGTGTCGAGA-CCGGGTAGC-TAATTTTATGC-CAAAAA------
ZK643(1)                        AACAAAAAAGTGTCGCTTCGAGA-CCGGGTA-CCGTGTTTTTGGCGCAAAAATCGCTAT
ZK643(2,for)                    ATAGAAAG-TTGTCGTTTCGAGA-CCGGACA-CCGTATTTTTGGCGCAAAATATACCTG
ZK643(2,rev)                    ACAGAAAA-TTCTCGTTTCGAGA-CCGAACA-CAGTATTTTTGGCGGAGAAATTCTAAA
ZK643(3)                        --------TTTGTCGTGTCGAGA-CCTGG-....................------
glp-1(for)                      AACAAATTTTTGTCGTGGCGAGA-CCTGATA-CCGTATTTTTAGGTCAAGATTACTAGG
glp-1(rev)                      -------GTTTGTCGT-..........................------
consensus                       A----A----T-TCG---CGAGA-CC------C-----TTT------A--A--------
```

FIGURE 2A(iii)

Repeat 4

	1 10 20 30 40 50 60 70
ced-3	AACCAATCAGCATCGTCGATCTCGGCCCACTTCATCGGATTGGTTTGAAAGTGGGCGGAGTGAATTGCTGATTGGTC
Lin-12	AACCAATTAGCGACTTCGGAATTTCCATACTTAATCTGATTGGTTGAAGAATGGGCAGAGCGAATTGCTGATTGGCC
consensus	AACCAAT-AGC--C-TCG---T---C--ACTT-ATC-GATTGGTT--A-A-TGGGC-GAG-GAATTGCTGATTGG-C

FIGURE 2A(iv)

Repeat 5

		10	20	30	40	50	60
	cad-3(for)	TTTTAAG-GACACAGAAAAATAGGCAGAGGCTCCTTTTGCAAGCCTGCCGCGCGTCAACC					
	cad-3(rev)	TTTCAAGCCGCACAGAAAAAGAGGCGGAGCGTCGTTTTGCAAACTTGCCGCGCGCCAACC					
5	consensus	TTT-AAG---CACAGAAAAA-AGGC-GAG--TC-TTTTGCAA-C-TGCCGCGCG-CAACC					

FIGURE 2A(v)

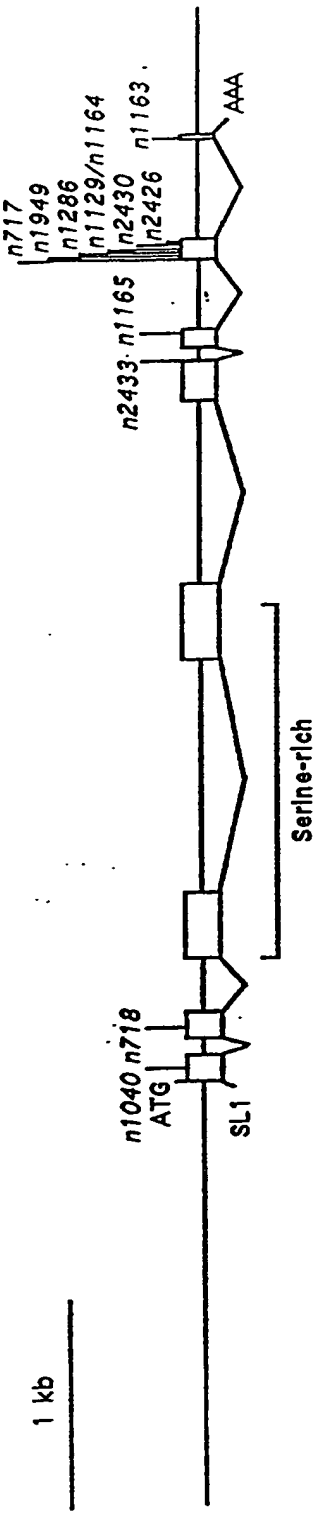
AAA
n1163
n1164
n1129/n1164
n1286
n1949
n717
n2426
n2430
n1165
n2433
Serine-rich
n718
n1040
ATG
SL1
1 kb

FIGURE 2B

10136

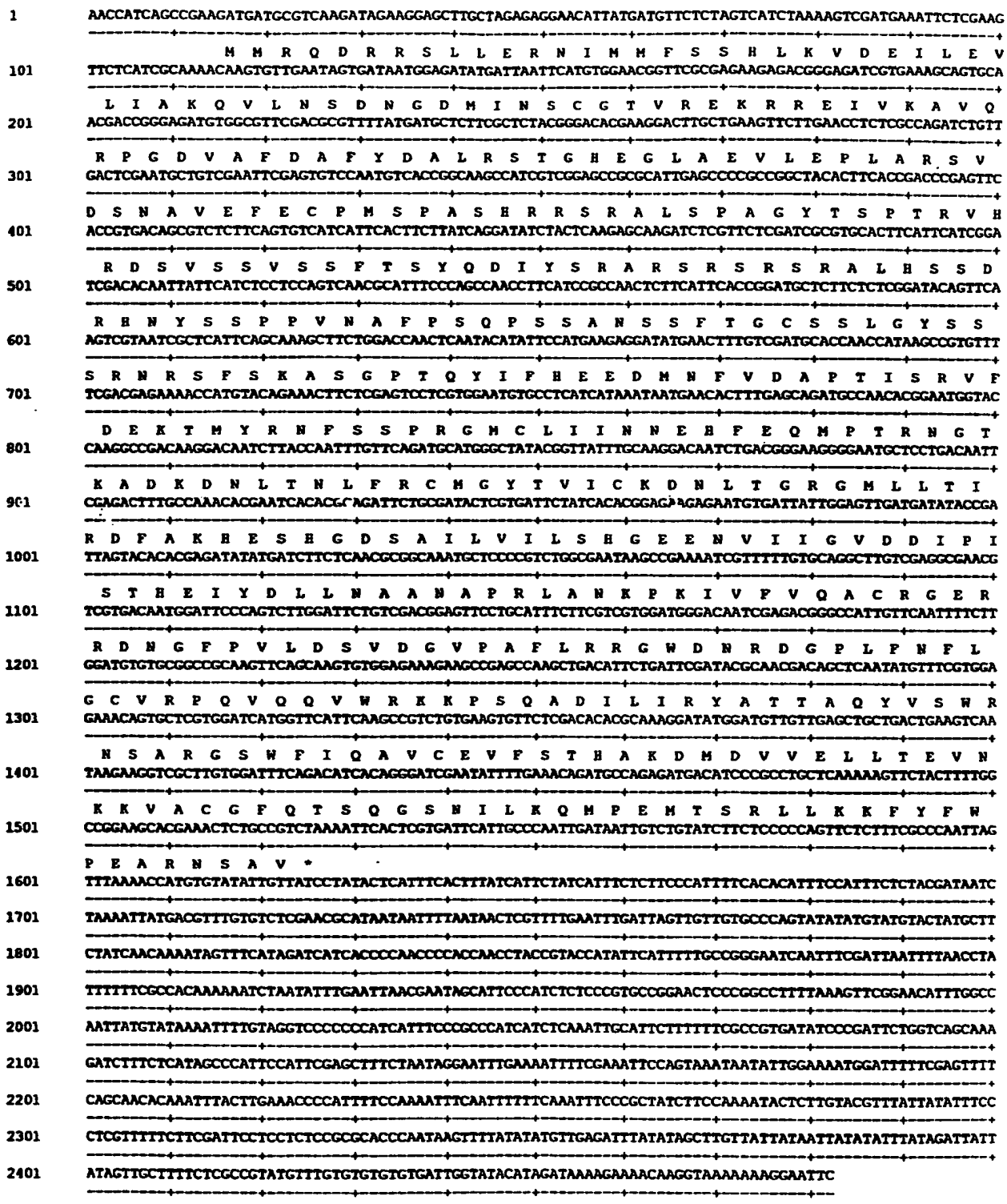

FIGURE 2C

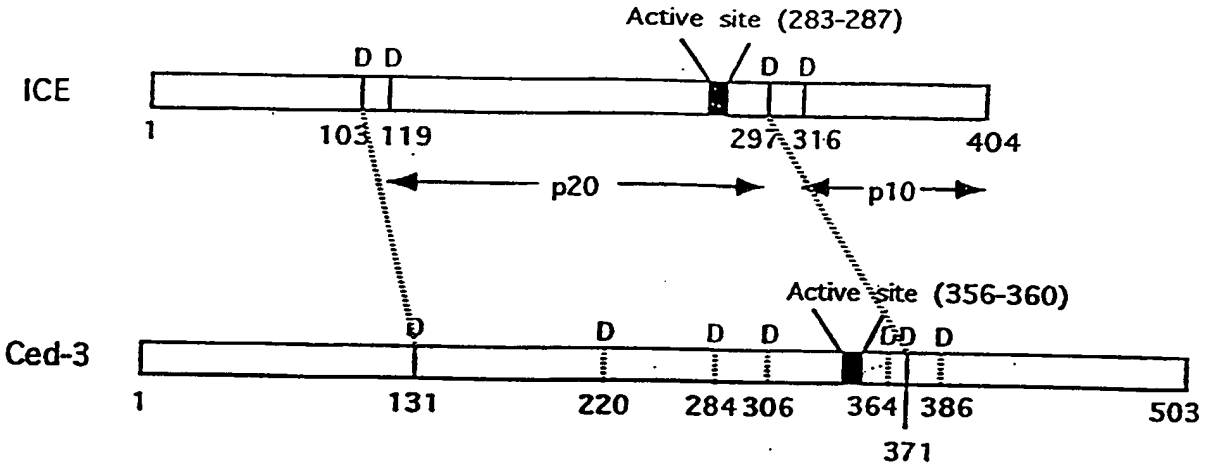
Active site (283-287)
ICE
D D
D D
1
103 119
297 316
404
p20
p10
Active site (356-360)
Ced-3
D
D
D
D
D D
D
1
131
220
284 306
364
386
503
371

FIGURE 3

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FIGURE 3A

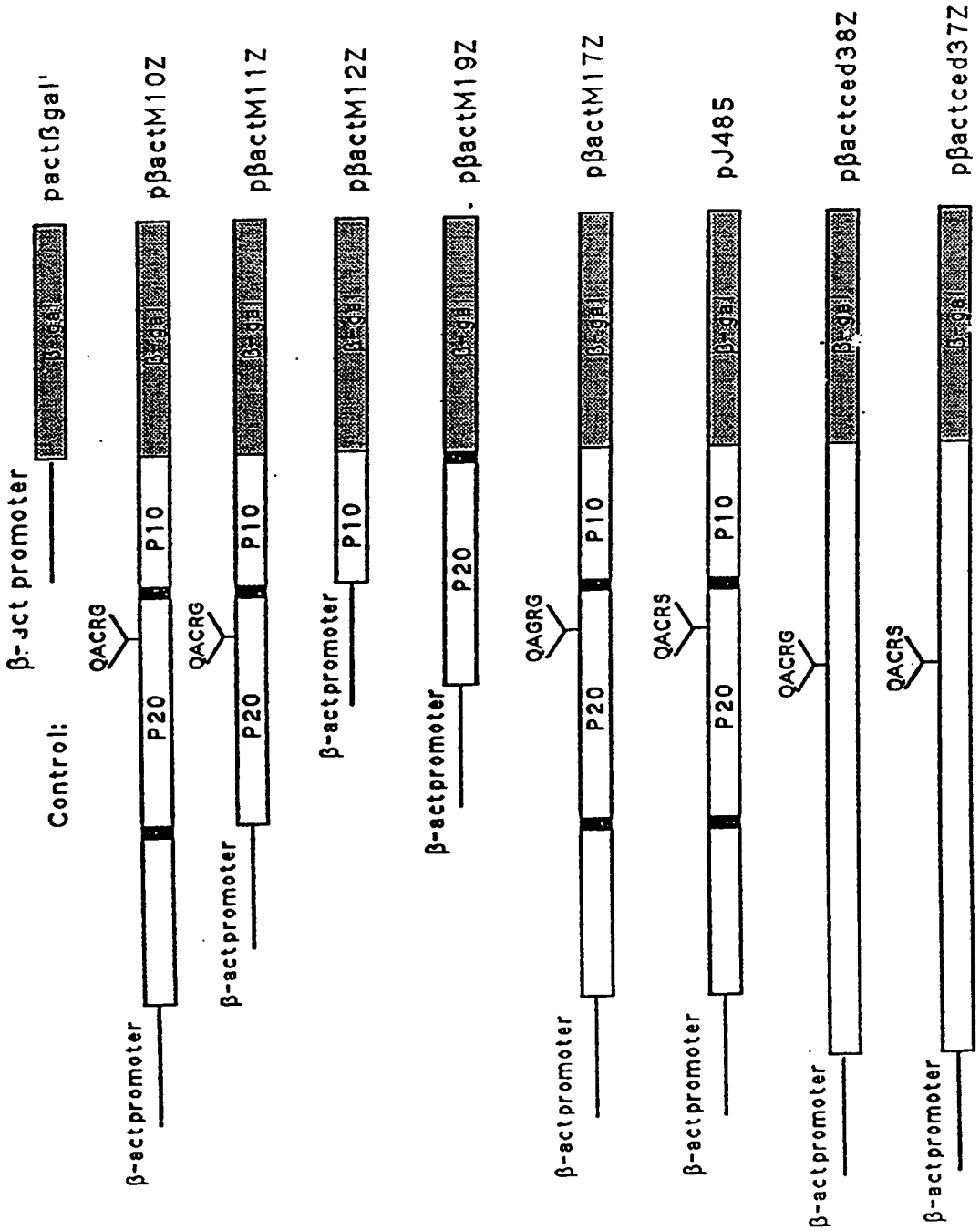
Control:
β-jct promoter
pactβgal'
β-actpromoter
QACRG
P20
P10
pβactM10Z
pβactM11Z
pβactM12Z
pβactM19Z
QAGRG
pβactM17Z
QACRS
pJ485
pβactced38Z
pβactced37Z

FIGURE 4

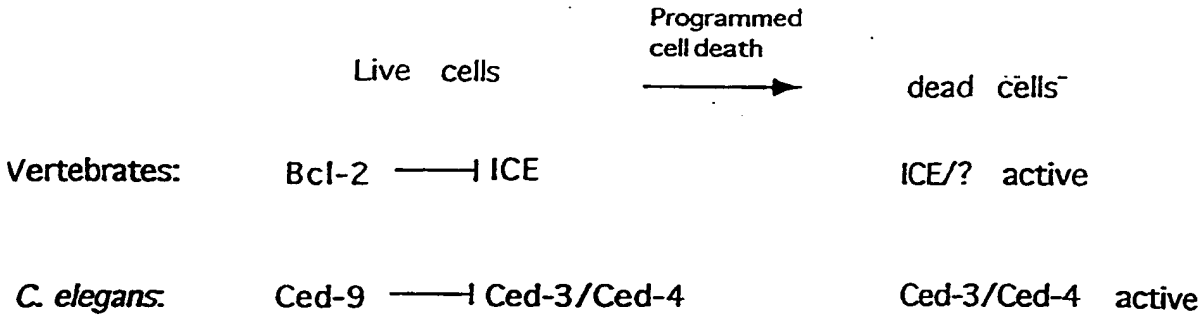
Programmed cell death
Live cells
dead cells
Vertebrates:
Bcl-2
ICE
ICE/? active
C. elegans:
Ced-9
Ced-3/Ced-4
Ced-3/Ced-4 active

FIGURE 5

15136

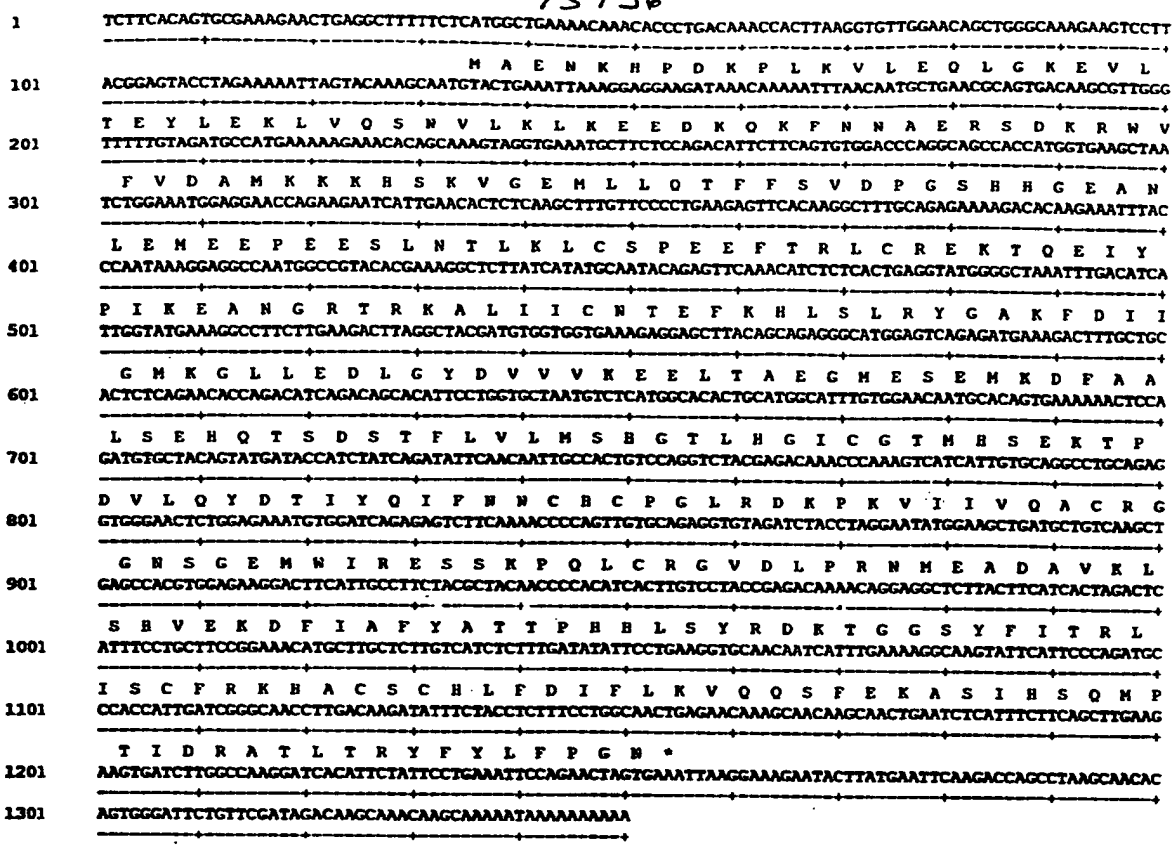

FIGURE 6

```
          1                                                  50
MICE1     MADKILRAKR KQFINS.... .VSIGTINGL LDELLEKRVL NQEEMDKIKL
HICE      MADKVLKEKR KLFIRS.... .MGEGTINGL LDELLQTRVL NKEEMEKVKR
MICE2     MAENKHPDKP LKVLEQ.... .LGKEVLTEY LEKLVQSNVL KLKEEDKQKF
Ced3      ....MMRQDR RSLLERNIMM FSSHLKVDEI LEVLIAKQVL NSDNGDMIN.

          51                                                 100
MICE1     ANITAMDKAR NLCDHVSKKG APASQ.IFIT YICNEDCYDA GLDELQSAPS
HICE      ENATVMDKCR ALLDSVIPKG AQACQ.ICIT YICEEDSYDA GTLGLSADQT
MICE2     NNAERSDKRW VFVDAMKKKH SKVGE.MLL. ..........-- ---------
Ced3      SCGTVREKRR EIVKAVQRPG DVAFDAFYDA LRSTGHEGDA EVLEPLARSV

          101                                                150
MICE1     AETFVATEDS KGGHPSSSET KE.EQNKEDG TFPGLTGTLK ECPLEKAQKL
HICE      SGNYLNMQDS QGVLSSFPAP QAVQDNPAMP TSSGSEGNVK LCSLEEAQRI
MICE2     .......... QTFFSVDPGS HHGEANLEME EPEESLNTLK LCSPEEFTRL
Ced3      DSNAVEFECP MSPASHRRSR ALSPAGYTSP TRVHRDSVSS VSSFTSYQDI

          151                                                200
MICE1     WKE..... .......... .......... .......... ..........
HICE      WKQ..... .......... .......... .......... ..........
MICE2     CRE..... .......... .......... .......... ..........
Ced3      YSRARSRSRS RALHSSDRHN YSSPPVNAFP SQPSSANSSF TGCSSLGYSS

          201                                                250
MICE1     .......... .......... .....NPSEI YPLMNTTTRT R.......LA
HICE      .......... .......... .....KSAEI YPLMDKSSRT R.......LA
MICE2     .......... .......... .....KTQEI YPLKEANGRT R.......KA
Ced3      SRNRSFSKAS GPTQYIFHEE DMNFVDAPTI SRVFDEKTMY RNFSSPRGMC

          251                                                300
MICE1     LLLQNLEFQH LSHRVGAQVD LREMKLLLED LGYQVKKVKN LTALEMVKEV
HICE      LLLQNEEFDS IPRRTGAEVD ITGMTMLLQN LGYSVDVKKN LTASDMTTEL
MICE2     LLLCNTEFKH LSIRYGAKFD IIGMKGLLED LGYDVVVKEE LTAEGMESEM
Ced3      LLLNNEHEEQ MPTRNGTKAD KDNLTNLFRC MGYNVICKDN LTGRGMLLTI

          301                                                350
MICE1     KEFAACPEHK LSDSTFLVFM SHGIQEGICG TTYSNEVSDI LKVDTIFQM
HICE      EAFAHRPEHK LSDSTFLVFM SHGIREGICG KKHSEQVPDI LQLNAIFNM
MICE2     KDFAALSEHQ LSDSTFLVLM SHGTLHGICG TMHSEKTPDV LQYDTIYQI
Ced3      RDFAKHESHL .GDSAILVIL SHGEENVIIG .....VDDI PISTHEIYDL

          351                                                400
MICE1     MNTLKCPSLK DKPKVIIIQA CRGEKQGVVL LKDSVRDSEE DFLTDAIFE
HICE      LNTKNCPSLK DKPKVIIIQA CRGDSPGVVW FKDSVGVSGN LSLPTTEEFE
MICE2     FNNCHCPGLR DKPKVIIIQA CRGGNSGEMW IRESSKPQLC RGVDLPRNME
Ced3      LNAANAPRLA NKPKIVFVQA CRGERRDNGF PVLDSVDGVP AFLRRGWDNR
```

FIGURE 7

```
          401                                                  450
MICE1     DDGI..... .........K KAHIEKDFIA FCSSTPDNVS WRHPVRGSLF
HICE      DDAI..... .........K KAHIEKDFIA FCSSTPDNVS WRHPTMGSVF
MICE2     ADAV..... .........K LSHVEKDFIA FYATTPHHLS YRDKTGGSYF
Ced3      DGPLFNFLGC VRPQVQQVWR KKPSQADILI RYATTAQYVS WRNSARGSWE

          451                                                  500
MICE1     IESLIKHMKE YAWSCDLEDI F....RKVRF SFEQPEFRLQ MPTADRVT..
HICE      IGRLIEHMQE YACSCDVEEI F....RKVRF SFEQPDGRAQ MPTTERVT..
MICE2     ITRLISCFRK HACSCHLFDI F....LKVQQ SFEKASIHSQ MPTIDRAT..
Ced3      IQAVCEVFST HAKDMDVVEL LTEVNKKVAC GEQTSQGSNI LKQMPEMTSR

          501        517
MICE1     LITKRFYLFP G H......
HICE      LITRCFYLFP G H......
MICE2     LITRYFYLFP G N*.....
Ced3      LLKKEYFWPE ARNSAV*
```

FIGURE 7A

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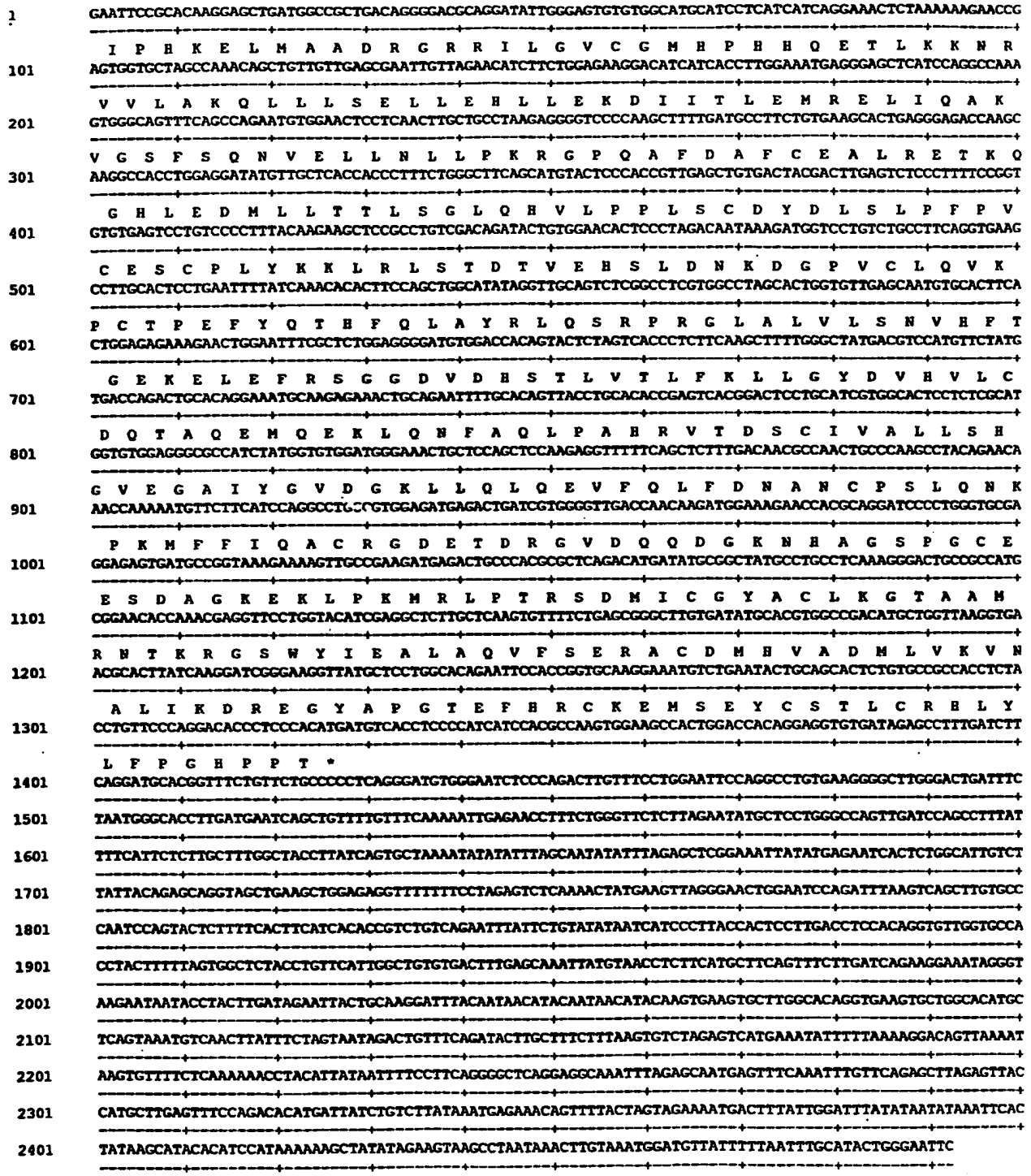
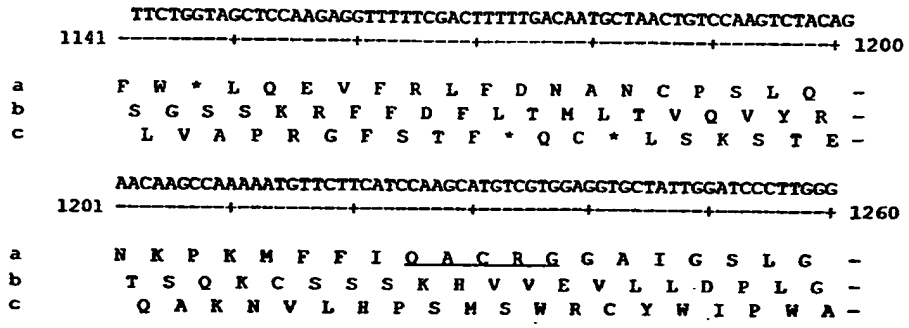


FIGURE 8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1141
1200
a F W * L Q E V F R L F D N A N C P S L Q -
b S G S S K R F F D F L T M L T V Q V Y R -
c L V A P R G F S T F * Q C * L S K S T E -
AACAAGCCAAAAATGTTCTTCATCCAAGCATGTCGTGGAGGTGCTATTGGATCCCTTGGG
1201
1260
a N K P K M F F I Q A C R G G A I G S L G -
b T S Q K C S S S K H V V E V L L D P L G -
c Q A K N V L H P S M S W R C Y W I P W A -

FIGURE 9

```
251 ATGAAAGTGCATTTTTATATCTTTATGCTGTACACTTCACTCACTGTAAT 300
  1 .............................GAATTCCGCACAAGGAGCTGA 21

301 GGTGGGTAAGTTTAGTGAGCAATAGGTCACTATGGTGACCCATGTCACAC 350
 22 TGGCCGCTGACAGGGGACGCAGGATATTGGGAGTGTGTGGCATGCATCCT 71

351 AGCCTGAATGTTATGGTAAAATATACTC......TGCTAAATAAATTAGT 394
 72 CATCATCAGGAAACTCTAAAAAAGAACCGAGTGGTGCTAGCCAAACAGCT 121

395 CCATTATTTAAATTCAACCTCAAGTTTTCAGAATATAGACGACTGCGTCC 444
122 GTTGTTGAGCGAATTGTTAGAACATCTTCTGGAGAAGGACATCATCACCT 171

445 AATTCCTCACCAAAGTAGGACACAAGCTGAGTTCTCATTAAAGTCCTTGA 494
172 TGGAAATGAGGGAGCTCATCCAGGCCAAAGTGGGCAGTTTCAGCCAGAAT 221

495 TCTGTTCTAGGATCTATTGAGCCTAGCCTGTGTCTTCATTTTTCTTAATT 544
222 GTGGAACTCCTCAACTTGCTGCCTAAGAGGGGTCCCCAAGCTTTTGATGC 271

545 AACACTTGGGTGTCTTGAAAACCCACTAAAATTGCCCCTGAAGCCCTGCT 594
272 CTTCTGTGAAGCACTGAGGGAGACCAAGCAAGGCCACCTGGAGGATATGT 321

595 TAGAGCATTCTGGGCTTTTTAGCCTGCAGCTTCTAATTCTCCCATATTCC 644
322 T...GCTCACCACCCTTTCTGGGCTTCAGCATGTACTCCCACCGTTGAGC 368

645 TCCAACAAACTAGTTCCAATGGCCTATGAACCATGTGGAAAGATTATGGC 694
369 TGTGACTACGACTTGAGTCTCCCTTTTCCGGTGTGTGAGTCCTGTCCCCT 418

695 TGTATTGACCCCAAACTGGCATAAGTTTTCAGTATTGGTTTTCTTGGACT 744
419 TTACAAGAAGCTCCGCCTGTCGACAGATACTGTGGAACACTCCCTAGACA 468

745 GAGACCAAAAATTCTTAACGAGAAACAGTTTGTTTTGGCTCCTTGTTTGA 794
469 ATAAAGATGGTCCTGTCTGCCTTCAGGTGAAGCCTTGCACTCCTGAATTT 518

795 CAATATAAGCCCCCGTGGCAAGAAGCCAGCTCTCCAGTGGAGACAGGAGC 844
519 TATCAAACACACTTCCAGCTGGCATATAGGTTGCAGTCTCGGCCTCGTGG 568

845 AGTAGGCTTCTTGTATTTGGGTAAATTGTGTAGCATAAAAGAAGAGATGC 894
569 CCTAGCACTGGTG...TTGAGCAATGTGCACTTCACTGGAGAGAAAGAAC 615

895 TGGCATGTCTGGCTTTCTTTGCCCCAACCCTTTCAGTCTGGGTTCCAGGT 944
616 TGGAATTTCGCTCTGGAGGGG..............ATGTGGACCACAGTA 651

945 CTTTCCTCCTCAGTTAATTCTTTCTGGAAATTCAATACCCTCAAAAATAA 994
652 CTCTAGTCACCCTCTTCAAGCTTTTGGGCTATGACGTCCATGTTCTATGT 701
```

FIGURE 10

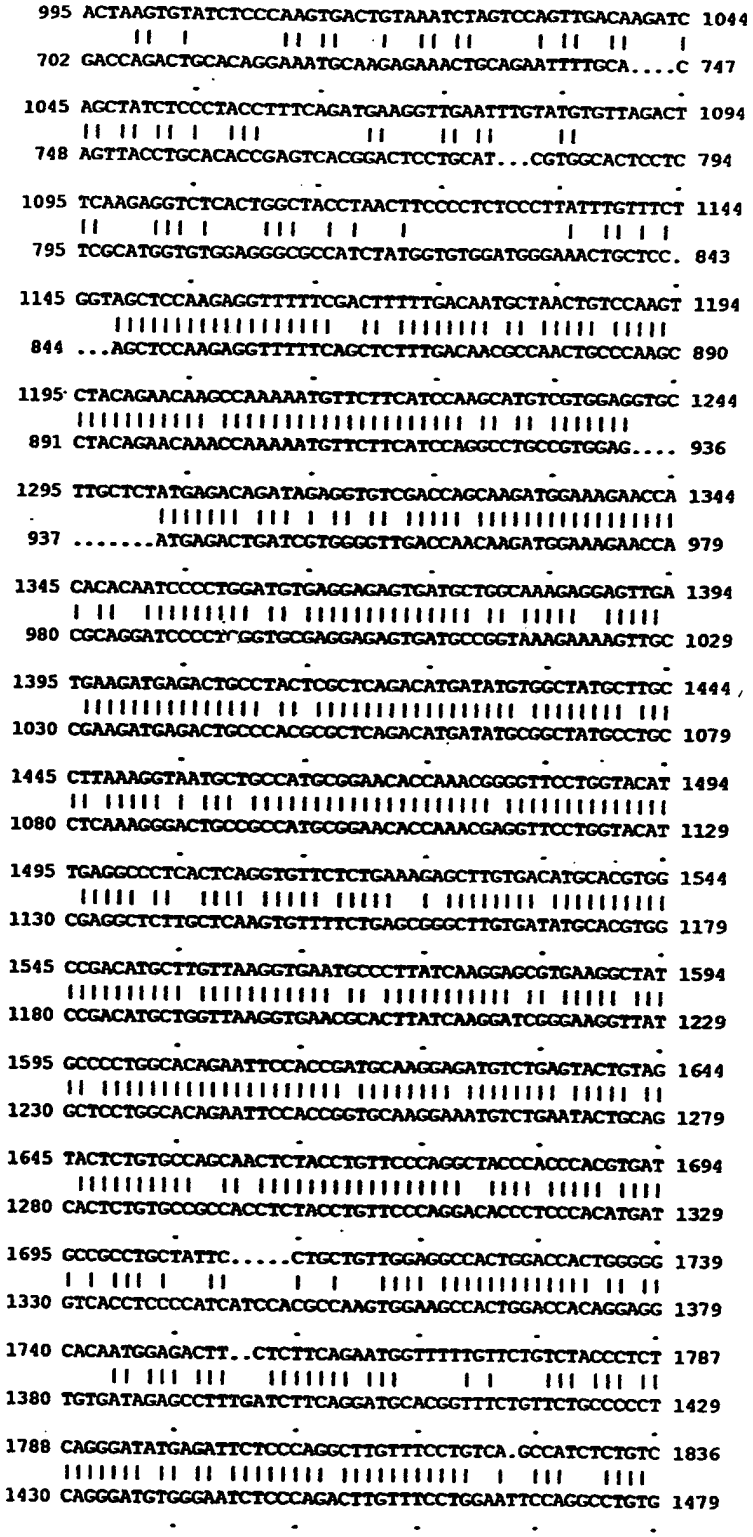
995 ACTAAGTGTATCTCCCAAGTGACTGTAAATCTAGTCCAGTTGACAAGATC 1044
702 GACCAGACTGCACAGGAAATGCAAGAGAAACTGCAGAATTTTGCA....C 747
1045 AGCTATCTCCCTACCTTTCAGATGAAGGTTGAATTTGTATGTGTTAGACT 1094
748 AGTTACCTGCACACCGAGTCACGGACTCCTGCAT...CGTGGCACTCCTC 794
1095 TCAAGAGGTCTCACTGGCTACCTAACTTCCCCTCTCCCTTATTTGTTTCT 1144
795 TCGCATGGTGTGGAGGGCGCCATCTATGGTGTGGATGGGAAACTGCTCC. 843
1145 GGTAGCTCCAAGAGGTTTTTCGACTTTTTGACAATGCTAACTGTCCAAGT 1194
844 ...AGCTCCAAGAGGTTTTTCAGCTCTTTGACAACGCCAACTGCCCAAGC 890
1195 CTACAGAACAAGCCAAAAATGTTCTTCATCCAAGCATGTCGTGGAGGTGC 1244
891 CTACAGAACAAACCAAAAATGTTCTTCATCCAGGCCTGCCGTGGAG.... 936
1295 TTGCTCTATGAGACAGATAGAGGTGTCGACCAGCAAGATGGAAAGAACCA 1344
937 .......ATGAGACTGATCGTGGGGTTGACCAACAAGATGGAAAGAACCA 979
1345 CACACAATCCCCTGGATGTGAGGAGAGTGATGCTGGCAAAGAGGAGTTGA 1394
980 CGCAGGATCCCCTGGTGCGAGGAGAGTGATGCCGGTAAAGAAAAGTTGC 1029
1395 TGAAGATGAGACTGCCTACTCGCTCAGACATGATATGTGGCTATGCTTGC 1444
1030 CGAAGATGAGACTGCCCACGCGCTCAGACATGATATGCGGCTATGCCTGC 1079
1445 CTTAAAGGTAATGCTGCCATGCGGAACACCAAACGGGGTTCCTGGTACAT 1494
1080 CTCAAAGGGACTGCCGCCATGCGGAACACCAAACGAGGTTCCTGGTACAT 1129
1495 TGAGGCCCTCACTCAGGTGTTCTCTGAAAGAGCTTGTGACATGCACGTGG 1544
1130 CGAGGCTCTTGCTCAAGTGTTTTCTGAGCGGGCTTGTGATATGCACGTGG 1179
1545 CCGACATGCTTGTTAAGGTGAATGCCCTTATCAAGGAGCGTGAAGGCTAT 1594
1180 CCGACATGCTGGTTAAGGTGAACGCACTTATCAAGGATCGGGAAGGTTAT 1229
1595 GCCCCTGGCACAGAATTCCACCGATGCAAGGAGATGTCTGAGTACTGTAG 1644
1230 GCTCCTGGCACAGAATTCCACCGGTGCAAGGAAATGTCTGAATACTGCAG 1279
1645 TACTCTGTGCCAGCAACTCTACCTGTTCCCAGGCTACCCACCCACGTGAT 1694
1280 CACTCTGTGCCGCCACCTCTACCTGTTCCCAGGACACCCTCCCACATGAT 1329
1695 GCCGCCTGCTATTC.....CTGCTGTTGGAGGCCACTGGACCACTGGGGG 1739
1330 GTCACCTCCCCATCATCCACGCCAAGTGGAAGCCACTGGACCACAGGAGG 1379
1740 CACAATGGAGACTT..CTCTTCAGAATGGTTTTTGTTCTGTCTACCCTCT 1787
1380 TGTGATAGAGCCTTTGATCTTCAGGATGCACGGTTTCTGTTCTGCCCCCT 1429
1788 CAGGGATATGAGATTCTCCCAGGCTTGTTTCCTGTCA.GCCATCTCTGTC 1836
1430 CAGGGATGTGGGAATCTCCCAGACTTGTTTCCTGGAATTCCAGGCCTGTG 1479

FIGURE 10A

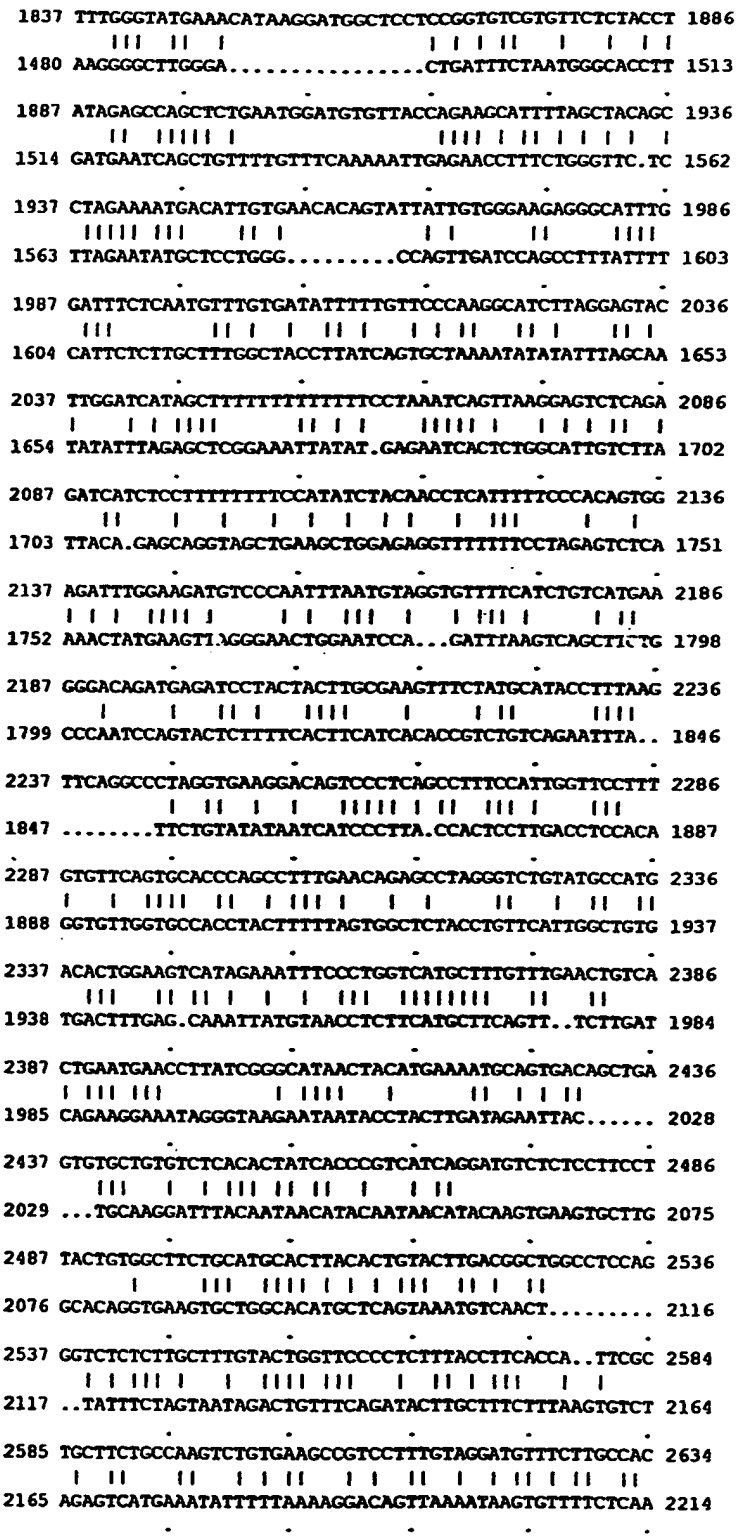
1837 TTTGGGTATGAAACATAAGGATGGCTCCTCCGGTGTCGTGTTCTCTACCT 1886
1480 AAGGGGCTTGGGA.....CTGATTTCTAATGGGCACCTT 1513
1887 ATAGAGCCAGCTCTGAATGGATGTGTTACCAGAAGCATTTTAGCTACAGC 1936
1514 GATGAATCAGCTGTTTTGTTTCAAAAATTGAGAACCTTTCTGGGTTC.TC 1562
1937 CTAGAAAATGACATTGTGAACACAGTATTATTGTGGGAAGAGGGCATTTG 1986
1563 TTAGAATATGCTCCTGGG.....CCAGTTGATCCAGCCTTTATTTT 1603
1987 GATTTCTCAATGTTTGTGATATTTTTGTTCCCAAGGCATCTTAGGAGTAC 2036
1604 CATTCTCTTGCTTTGGCTACCTTATCAGTGCTAAAATATATATTTAGCAA 1653
2037 TTGGATCATAGCTTTTTTTTTTTTTCCTAAATCAGTTAAGGAGTCTCAGA 2086
1654 TATATTTAGAGCTCGGAAATTATAT.GAGAATCACTCTGGCATTGTCTTA 1702
2087 GATCATCTCCTTTTTTTTCCATATCTACAACCTCATTTTTCCCACAGTGG 2136
1703 TTACA.GAGCAGGTAGCTGAAGCTGGAGAGGTTTTTTTCCTAGAGTCTCA 1751
2137 AGATTTGGAAGATGTCCCAATTTAATGTAGGTGTTTTCATCTGTCATGAA 2186
1752 AAACTATGAAGTTAGGGAACTGGAATCCA...GATTTAAGTCAGCTTCTG 1798
2187 GGGACAGATGAGATCCTACTACTTGCGAAGTTTCTATGCATACCTTTAAG 2236
1799 CCCAATCCAGTACTCTTTTCACTTCATCACACCGTCTGTCAGAATTTA.. 1846
2237 TTCAGGCCCTAGGTGAAGGACAGTCCCTCAGCCTTTCCATTGGTTCCTTT 2286
1847 ........TTCTGTATATAATCATCCCTTA.CCACTCCTTGACCTCCACA 1887
2287 GTGTTCAGTGCACCCAGCCTTTGAACAGAGCCTAGGGTCTGTATGCCATG 2336
1888 GGTGTTGGTGCCACCTACTTTTTAGTGGCTCTACCTGTTCATTGGCTGTG 1937
2337 ACACTGGAAGTCATAGAAATTTCCCTGGTCATGCTTTGTTTGAACTGTCA 2386
1938 TGACTTTGAG.CAAATTATGTAACCTCTTCATGCTTCAGTT..TCTTGAT 1984
2387 CTGAATGAACCTTATCGGGCATAACTACATGAAAATGCAGTGACAGCTGA 2436
1985 CAGAAGGAAATAGGGTAAGAATAATACCTACTTGATAGAATTAC..... 2028
2437 GTGTGCTGTGTCTCACACTATCACCCGTCATCAGGATGTCTCTCCTTCCT 2486
2029 ...TGCAAGGATTTACAATAACATACAATAACATACAAGTGAAGTGCTTG 2075
2487 TACTGTGGCTTCTGCATGCACTTACACTGTACTTGACGGCTGGCCTCCAG 2536
2076 GCACAGGTGAAGTGCTGGCACATGCTCAGTAAATGTCAACT..... 2116
2537 GGTCTCTCTTGCTTTGTACTGGTTCCCCTCTTTACCTTCACCA..TTCGC 2584
2117 ..TATTTCTAGTAATAGACTGTTTCAGATACTTGCTTTCTTTAAGTGTCT 2164
2585 TGCTTCTGCCAAGTCTGTGAAGCCGTCCTTTGTAGGATGTTTCTTGCCAC 2634
2165 AGAGTCATGAAATATTTTTAAAAGGACAGTTAAAATAAGTGTTTTCTCAA 2214

FIGURE 10B

```
2635 TTACGCTGTACTGTAGTTGCTTATTCTTTCTGCCTTCTGCTTCAGCGTGA 2684
2215 AAAACCTACATTATAAT.........TTTCCTTCAGGGGCTCAGGAGGCA 2255
2685 GGCTTCTTTGGTTTTCTGTGGCAGCGTCTCCCTTCTCATTGTTTCTCTG. 2733
2256 AATTTAGAGCAATGAGTTTCAAATTTGTTCAGAGCTTAGAGTTACCATGC 2305
2734 .TGTTTTAGTGGGGATAGTACCATATGTGATATAACCTAGAAGCACTTGT 2782
2306 TTGAGTTTCCAGACACATGATTATCTGTCTTATAAATGAGAAACAGTTTT 2355
2783 CTCTGCTCTTATGAAACTTGCTTATTCTTGAAAACCTTCTGCATTTCCAT 2832
2356 ACTAGTAGAAAATGACTTTATTGGATTTATATAATATAAATTCACTATAA 2405
2833 TTTTTCCTCTCTTCCAATTTATTCTCCATGTAACAGAGTAGTTTGGTTTT 2882
2406 GCATACACATCCATAAAAAAGCTATATAGAAGTAAGCCTAATAAACTTGT 2455
2883 TAAAATATCTGGTGATGTCATTCTCTTGCTTAGAACACTAGCTTCCTGTT 2932
2456 AAATGGATGTTAT.TTTTAATTTGCATACTGGGAATTC..... 2492
```

FIGURE 10C

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```
Ced-3   1 ....................MMRQORRSLLERNIMMFSSHLKVDEILEVLI  31
                               |:..:.. |.:| :::..:| :.|:|| |:
ICE3    1 IPHKELMAADRGRRILGVCGMHPHHQETLKKNRVVLAKQLLLSELLEHLL  50

Ced-3  32 AKQVLNSDNGDMINSCGTVREKRREIVKAVQRPGDVAFDAFYDALRSTGH  81
          .|:::. :  ::|.. ..  ... |::. :.:.|. ||||||:|||.| :
ICE3   51 EKDIITLEMRELIQAKVGSFSQNVELLNLLPKRGPQAFDAFCEALRETKQ 100

Ced-3  82 EGLAEVLEPLARSVDSNAVEFECPMSPASHRRSRALSPAGYTSPTRVHRD 131
          : |.::|
ICE3  101 GHLEDML..................................................  107

Ced-3 132 SVSSVSSFTSYQDIYSRARSRSRSRALHSSDRHNYSSPPVNAFPSQPSSA 181
             :..:.:.|.:                                :|. .:.
ICE3  108 ....LTTLSGLQHV..............................LPPLSCDY 125

Ced-3 182 NSSFTGCSSLGYSSSRNRSFSKASGPTQYIFHEEDMNFVDAPTISRVFDE 231
          : |:. . : ::.  :. .:|.....  . .::.: :   |... .::.
ICE3  126 DLSLPFPVCESCPLYKKLRLSTDTVERSLDNKDGPVCLQVKPCTPEFYQT 175

Ced-3 232 ..KTMYRNFSSPRGMCLIINNEHF...EQMPTRNGTKADKDNLTNLFRCM 276
            . || |.|||:.|::.| ||   .::. |.|...|...|..||: :
ICE3  176 HFQLAYRLQSRPRGLALVLSNVHFTGEKELEFRSGGDVDHSTLVTLFKLL 225

Ced-3 277 GYTVICKDNLTGRGMLLTIRDFAKHESH..GDSAILVILSHGEENVIIGV 324
            ||.| .  : |:.:|  .:.:||. ..|  .||.|:.:|||| |..|.||
ICE3  226 GYDVHVLCDQTAQEMQEKLQNFAQLPAHRVTDSCIVALLSHGVEGAIYGV 275

Ced-3 325 DDIPISTHEIYDLLNAANAPRLANKPKIVFVQACRGERRDNGFPVLDSVD 374
          |:  :  :|:::|::.||.|.|.||||:.|:|||||:  |.|..  |
ICE3  276 DGKLLQLQEVFQLFDNANCPSLQNKPKMFFIQACRGDETDRGVDQQD... 322

Ced-3 375 GVPAFLRRGWDNRDGPLFNFLGCVRPQVQQVWRKKPSQADILIRYATTAQ 424
                |.:: ::|  :  :..:..:..::   |...|::. ||.  .
ICE3  323 ........GKNHAGSPGCEESDAGKEKLPKMR..LPTRSDMICGYACLKG 362

Ced-3 425 YVSWRNSARGSWFIQAVCEVFSTHAKDMDVVELLTEVNK..KVACGFQTS 472
          .. ||. ||||:|:|:.:|||.:| ||.|.::|..||   |   |:..:
ICE3  363 TAAMRNTKRGSWYIEALAQVFSERACDMHVADMLVKVNALIKDREGYAPG 412

Ced-3 473 QGSNILKQMPEMTSRLLKKFYFWPEARNSAV* 504
          : :  |:|.| .| | :.:|::|: . .
ICE3  413 TEFHRCKEMSEYCSTLCRHLYLFPGHPPT*.. 442
```

FIGURE 11

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```
ICE     1 .....MADKVLKEKRKLFIRSMGEGTINGLLD 27
ICE3    1 IPHKELMAADRGRRILGVCGMHPHHQETLKKNRVVLAKQL...LLSELLE 47

ICE    28 ELLQTRVLNKEEMEKVKRENATVMDKTRALIDSVIPKGAQACQICITYIC 77
ICE3   48 HLLEKDIITL.EMRELIQAKVGSFSQNVELLNLLPKRGPQAFDAFCEALR 96

ICE    78 E.EDSYLAGTLGLSADQTSGNYLNMQDSQGVLSSFPAPQAVQDNPAMPTS 126
ICE3   97 ETKQGHLEDML..........LTTLSGLQHVLPPLSCDYDLSLPFPVCES 136

ICE   127 SGSEGNVKLCSLEEAQRIWKQKSAEIYPIMDKS.................S 160
ICE3  137 CPLYKKLRL.STDTVEHSLDNKDGPVCLQVKPCTPEFYQTHFQLAYRLQS 185

ICE   161 RTR.LALIICNEEFDS...IPRRTGAEVDITGMTMLLQNLGYSVDVKKNL 206
ICE3  186 RPRGLALVLSNVHFTGEKELEFRSGGDVDHSTLVTLFKLLGYDVHVLCDQ 235

ICE   207 TASDMTTELEAFAHRPEHKTSDSTFLVFMSHGIREGICGKKHSEQVPDIL 256
ICE3  236 TAQEMQEKLQNFAQLPAHRVTDSCIVALLSHGVEGAIYGVD.....GKLL 280

ICE   257 QLNAIFNMLNTKNCPSLKDKPKVIIIQACRGDSPGVVWFKDSVGVSGNLS 306
ICE3  281 QLQEVFQLFDNANCPSLQNKPKMFFIQACRGDETD..RGVDQQDGKNHAG 328

ICE   307 LPTTEEFEDDAIK....KAHIEKDFIAFCSSTPDNVSWRHPTMGSVFIGR 352
ICE3  329 SPGCEESDAGKEKLPKMRLPTRSDMICGYACLKGTAAMRNTKRGSWYIEA 378

ICE   353 LIEHMQEYACSCDVEEIFRKVRFSFEQPDGRAQMPTTERV.........TL 394
ICE3  379 LAQVFSERACDMHVADMLVKVNALIKDREGYAPGTEFHRCKEMSEYCSTL 428

ICE   395 TRCFYLFPGH.... 404
ICE3  429 CRHLYLFPGHPPT* 442
```

FIGURE 11A

```
1       GCACAAGGAGCTGATGGCCGCTGACAGGGGACGCAGGATATTGGGAGTGTGTGGCATGCATCCTCATCATCAGGAAACTC
                  M  A  A  D  R  G  R  R  I  L  G  V  C  G  M  H  P  H  H  Q  E  T  L
81      TAAAAAAGAACCGAGTGGTGCTAGCCAAACAGCTGTTGTTGAGCGAATTGTTAGAACATCTTCTGGAGAAGGACATCATC
          K  K  N  R  V  V  L  A  K  Q  L  L  L  S  E  L  L  E  H  L  L  E  K  D  I  I
161     ACCTTGGAAATGAGGGAGCTCATCCAGGCCAAAGTGGGCAGTTTCAGCCAGAATGTGGAACTCCTCAACTTGCTGCCTAA
        T  L  E  M  R  E  L  I  Q  A  K  V  G  S  F  S  Q  N  V  E  L  L  N  L  L  P  K
241     GAGGGGTCCCCAAGCTTTTGATGCCTTCTGTGAAGCACTGAGGGAGACCAAGCAAGGCCACCTGGAGGATATGTTGCTCA
         R  G  P  Q  A  F  D  A  F  C  E  A  L  R  E  T  K  Q  G  H  L  E  D  M  L  L  T
321     CCACCCTTTCTGGGCTTCAGCATGTACTCCCACCGTTGAGCTGTGACTACGACTTGAGTCTCCCTTTTCCGGTGTGTGAG
          T  L  S  G  L  Q  H  V  L  P  P  L  S  C  D  Y  D  L  S  L  P  F  P  V  C  E
401     TCCTGTCCCCTTTACAAGAAGCTCCGCCTGTCGACAGATACTGTGGAACACTCCCTAGACAATAAAGATGGTCCTGTCTG
        S  C  P  L  Y  K  K  L  R  L  S  T  D  T  V  E  H  S  L  D  N  K  D  G  P  V  C
481     CCTTCAGGTGAAGCCTTGCACTCCTGAATTTTATCAAACACACTTCCAGCTGGCATATAGGTTGCAGTCTCGGCCTCGTG
         L  Q  V  K  P  C  T  P  E  F  Y  Q  T  H  F  Q  L  A  Y  R  L  Q  S  R  P  R  G
561     GCCTAGCACTGGTGTTGAGCAATGTGCACTTCACTGGAGAGAAAGAACTGGAATTTCGCTCTGGAGGGGATGTGGACCAC
          L  A  L  V  L  S  N  V  H  F  T  G  E  K  E  L  E  F  R  S  G  G  D  V  D  H
641     AGTACTCTAGTCACCCTCTTCAAGCTTTTGGGCTATGACGTCCATGTTCTATGTGACCAGACTGCACAGGAAATGCAAGA
        S  T  L  V  T  L  F  K  L  L  G  Y  D  V  H  V  L  C  D  Q  T  A  Q  E  M  Q  E
721     GAAACTGCAGAATTTTGCACAGTTACCTGCACACCGAGTCACGGACTCCTGCATCGTGGCACTCCTCTCGCATGGTGTGG
         K  L  Q  N  F  A  Q  L  P  A  H  R  V  T  D  S  C  I  V  A  L  L  S  H  G  V  E
801     AGGGCGCCATCTATGGTGTGGATGGGAAACTGCTCCAGCTCCAAGAGGTTTTTCAGCTCTTTGACAACGCCAACTGCCCA
          G  A  I  Y  G  V  D  G  K  L  L  Q  L  Q  E  V  F  Q  L  F  D  N  A  N  C  P
881     AGCCTACAGAACAAACCAAAAATGTTCTTCATCCAGGCCTGCCGTGGAGATGAGACTGATCGTGGGGTTGACCAACAAGA
        S  L  Q  N  K  P  K  M  F  F  I  Q  A  C  R  G  D  E  T  D  R  G  V  D  Q  Q  D
961     TGGAAAGAACCACGCAGGATCCCCTGGGTGCGAGGAGAGTGATGCCGGTAAAGAAAAGTTGCCGAAGATGAGACTGCCCA
          G  K  N  H  A  G  S  P  G  C  E  E  S  D  A  G  K  E  K  L  P  K  M  R  L  P  T
1041    CGCGCTCAGACATGATATGCGGCTATGCCTGCCTCAAAGGGACTGCCGCCATGCGGAACACCAAACGAGGTTCCTGGTAC
          R  S  D  M  I  C  G  Y  A  C  L  K  G  T  A  A  M  R  N  T  K  R  G  S  W  Y
1121    ATCGAGGCTCTTGCTCAAGTGTTTTCTGAGCGGGCTTGTGATATGCACGTGGCCGACATGCTGGTTAAGGTGAACGCACT
        I  E  A  L  A  Q  V  F  S  E  R  A  C  D  M  H  V  A  D  M  L  V  K  V  N  A  L
1201    TATCAAGGATCGGGAAGGTTATGCTCCTGGCACAGAATTCCACCGGTGCAAGGAAATGTCTGAATACTGCAGCACTCTGT
         I  K  D  R  E  G  Y  A  P  G  T  E  F  H  R  C  K  E  M  S  E  Y  C  S  T  L  C
1281    GCCGCCACCTCTACCTGTTCCCAGGACACCCTCCCACATGATGTCACCTCCCCATCATCCACGCCAAGTGGAAGCCACTG
          R  H  L  Y  L  F  P  G  H  P  P  T  *
1361    GACCACAGGAGGTGTGATAGAGCCTTTGATCTTCAGGATGCACGGTTTCTGTTCTGCCCCCTCAGGGATGTGGGAATCTC
1441    CCAGACTTGTTTCCTGGAATTCCAGGCCTGTGAAGGGGCTTGGGACTGATTTCTAATGGGCACCTTGATGAATCAGCTGT
1521    TTTGTTTCAAAAATTGAGAACCTTTCTGGGTTCTCTTAGAATATGCTCCTGGGCCAGTTGATCCAGCCTTTATTTTCATT
```

Figure 12A

```
1601  CTCTTGCTTTGGCTACCTTATCAGTGCTAAAATATATATTTAGCAATATATTTAGAGCTCGGAAATTATATGAGAATCAC
1681  TCTGGCATTGTCTTATTACAGAGCAGGTAGCTGAAGCTGGAGAGGTTTTTTTCCTAGAGTCTCAAAACTATGAAGTTAGG
1761  GAACTGGAATCCAGATTTAAGTCAGCTTGTGCCCAATCCAGTACTCTTTTCACTTCATCACACCGTCTGTCAGAATTTAT
1841  TCTGTATATAATCATCCCTTACCACTCCTTGACCTCCACAGGTGTTGGTGCCACCTACTTTTTAGTGGCTCTACCTGTTC
1921  ATTGGCTGTGTGACTTTGAGCAAATTATGTAACCTCTTCATGCTTCAGTTTCTTGATCAGAAGGAAATAGGGTAAGAATA
2001  ATACCTACTTGATAGAATTACTGCAAGGATTTACAATAACATACAATAACATACAAGTGAAGTGCTTGGCACAGGTGAAG
2081  TGCTGGCACATGCTCAGTAAATGTCAACTTATTTCTAGTAATAGACTGTTTCAGATACTTGCTTTCTTTAAGTGTCTAGA
2161  GTCATGAAATATTTTTAAAAGGACAGTTAAAATAAGTGTTTTCTCAAAAAACCTACATTATAATTTTCCTTCAGGGGCTC
2241  AGGAGGCAAATTTAGAGCAATGAGTTTCAAATTTGTTCAGAGCTTAGAGTTACCATGCTTGAGTTTCCAGACACATGATT
2321  ATCTGTCTTATAAATGAGAAACAGTTTTACTAGTAGAAAATGACTTTATTGGATTTATATAATATAAATTCACTATAAGC
2401  ATACACATCCATAAAAAAGCTATATAGAAGTAAGCCTAATAAACTTGTAAATGGATGTTATTTTTAATTTGCATACTGGG
2481  AATTC
```

Figure 12A (cont'd)

```
1       AGAGGGAGGGAACGATTTAAGGAGCGAATACTACTGGTAAACTAATGGAAGAAATCTGCTGCACCACTGGATATTGGGAG
81      TGTGTGGCATGCATCCTCATCATCAGGAAACTCTAAAAAAGAACCGAGTGGTGCTAGCCAAACAGCTGTTGTTGAGCGAA
                M  H  P  H  H  Q  E  T  L  K  K  N  R  V  V  L  A  K  Q  L  L  L  S  E
161     TTGTTAGAACATCTTCTGGAGAAGGACATCATCACCTTGGAAATGAGGGAGCTCATCCAGGCCAAAGTGGGCAGTTTCAG
        L  L  E  H  L  L  E  K  D  I  I  T  L  E  M  R  E  L  I  Q  A  K  V  G  S  F  S
241     CCAGAATGTGGAACTCCTCAACTTGCTGCCTAAGAGGGGTCCCCAAGCTTTTGATGCCTTCTGTGAAGCACTGAGGGAGA
         Q  N  V  E  L  L  N  L  L  P  K  R  G  P  Q  A  F  D  A  F  C  E  A  L  R  E  T
321     CCAAGCAAGGCCACCTGGAGGATATGTTGCTCACCACCCTTTCTGGGCTTCAGCATGTACTCCCACCGTTGAGCTGTGAC
          K  Q  G  H  L  E  D  M  L  L  T  T  L  S  G  L  Q  H  V  L  P  P  L  S  C  D
401     TACGACTTGAGTCTCCCTTTTCCGGTGTGTGAGTCCTGTCCCCTTTACAAGAAGCTCCGCCTGTCGACAGATACTGTGGA
        Y  D  L  S  L  P  F  P  V  C  E  S  C  P  L  Y  K  K  L  R  L  S  T  D  T  V  E
481     ACACTCCCTAGACAATAAAGATGGTCCTGTCTGCCTTCAGGTGAAGCCTTGCACTCCTGAATTTTATCAAACACACTTCC
         H  S  L  D  N  K  D  G  P  V  C  L  Q  V  K  P  C  T  P  E  F  Y  Q  T  H  F  Q
561     AGCTGGCATATAGGTTGCAGTCTCGGCCTCGTGGCCTAGCACTGGTGTTGAGCAATGTGCACTTCACTGGAGAGAAAGAA
          L  A  Y  R  L  Q  S  R  P  R  G  L  A  L  V  L  S  N  V  H  F  T  G  E  K  E
641     CTGGAATTTCGCTCTGGAGGGGATGTGGACCACAGTACTCTAGTCACCCTCTTCAAGCTTTTGGGCTATGACGTCCATGT
        L  E  F  R  S  G  G  D  V  D  H  S  T  L  V  T  L  F  K  L  L  G  Y  D  V  H  V
721     TCTATGTGACCAGACTGCACAGGAAATGCAAGAGAAACTGCAGAATTTTGCACAGTTACCTGCACACCGAGTCACGGACT
         L  C  D  Q  T  A  Q  E  M  Q  E  K  L  Q  N  F  A  Q  L  P  A  H  R  V  T  D  S
801     CCTGCATCGTGGCACTCCTCTCGCATGGTGTGGAGGGCGCCATCTATGGTGTGGATGGGAAACTGCTCCAGCTCCAAGAG
          C  I  V  A  L  L  S  H  G  V  E  G  A  I  Y  G  V  D  G  K  L  L  Q  L  Q  E
881     GTTTTTCAGCTCTTTGACAACGCCAACTGCCCAAGCCTACAGAACAAACCAAAAATGTTCTTCATCCAGGCCTGCCGTGG
        V  F  Q  L  F  D  N  A  N  C  P  S  L  Q  N  K  P  K  M  F  F  I  Q  A  C  R  G
961     AGGTGCTATTGGATCCCTTGGGCACCTCCTTCTGTTCACTGCTGCCACCGCCTCTCTTGCTCTATGAGACTGATCGTGGG
         G  A  I  G  S  L  G  H  L  L  L  F  T  A  A  T  A  S  L  A  L  *
1041    GTTGACCAACAAGATGGAAAGAACCACGCAGGATCCCCTGGGTGCGAGGAGAGTGATGCCGGTAAAGAAAAGTTGCCGAA
1121    GATGAGACTGCCCACGCGCTCAGACATGATATGCGGCTATGCCTGCCTCAAAGGGACTGCCGCCATGCGGAACACCAAAC
1201    GAGGTTCCTGGTACATCGAGGCTCTTGCTCAAGTGTTTTCTGAGCGGGCTTGTGATATGCACGTGGCCGACATGCTGGTT
1281    AAGGTGAACGCACTTATCAAGGATCGGGAAGGTTATGCTCCTGGCACAGAATTCCACCGGTGCAAGGAGATGTCTGAATA
1321    CTGCAGCACTCTGTGCCGCCACCTCTACCTGTTCCCAGGACACCCTCCCACATGATGTCACCTCCCCATCATCCACGCCA
1401    AGTGGAAGCCACTGGACCACAGGAGGTGTGATAGAGCCTTTGATCTTCAGGATGCACGGTTTCTGTTCTGCCCCCTCAGG
1481    GATGTGGGAATCTCCCAGACTTGTTTCCTGTGCCCATCATCTCTGCCTTTGAGTGTGGGACTCCAGGCCAGCTCCTTTTC
1561    TGTGAAGCCCTTTGCCTGTAGAGCCAGCCTTGGTTGGACCTATTGCCAGGAATGTTTCAGCTGCAGTTGAAGAGCCTGAC
1641    AAGTGAAGTTGTAAACACAGTGTGGTTATGGGGAGAGGGCATATAAATTCCCCATATTTGTGTTCAGTTCCAGCTTTTGT
1721    AGATGGCACTTTAGTGATTGCTTTTATTACATTAGTTAAGATGTCTTGAGAGACCATCTCCTATCTTTTATTTCATTCAT
```

Figure 12B

```
1801    ATCCTCCGCCCTTTTTGTCCTAGAGTGAGAGTTTGGAAGGTGTCCAAATTTAATGTAGACATTATCTTTTGGCTCTGAAG
        ---------+---------+---------+---------+---------+---------+---------+---------+
1881    AAGCAAACATGACTAGAGACGCACCTTGCTGCAGTGTCCAGAAGCGGCCTGTGCGTTCCCTTCAGTACTGCAGCGCCACC
        ---------+---------+---------+---------+---------+---------+---------+---------+
1961    CAGTGGAAGGACACTCTTGGCTCGTTTGGGCTCAAGGCACCGCAGCCTGTCAGCCAACATTGCCTTGCATTTGTACCTTA
        ---------+---------+---------+---------+---------+---------+---------+---------+
2041    TTGATCTTTGCCCATGGAAGTCTCAAAGATCTTTCGTTGGTTGTTTCTCTGAGCTTTGTTACTGAAATGAGCCTCGTGGG
        ---------+---------+---------+---------+---------+---------+---------+---------+
2121    GAGCATCGGAATTC
        ---------+----
```

Figure 12B (cont'd)

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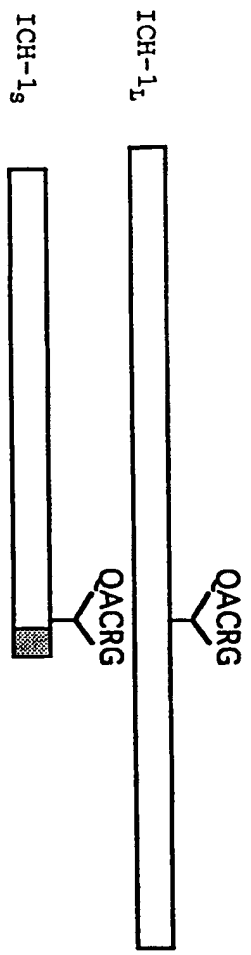
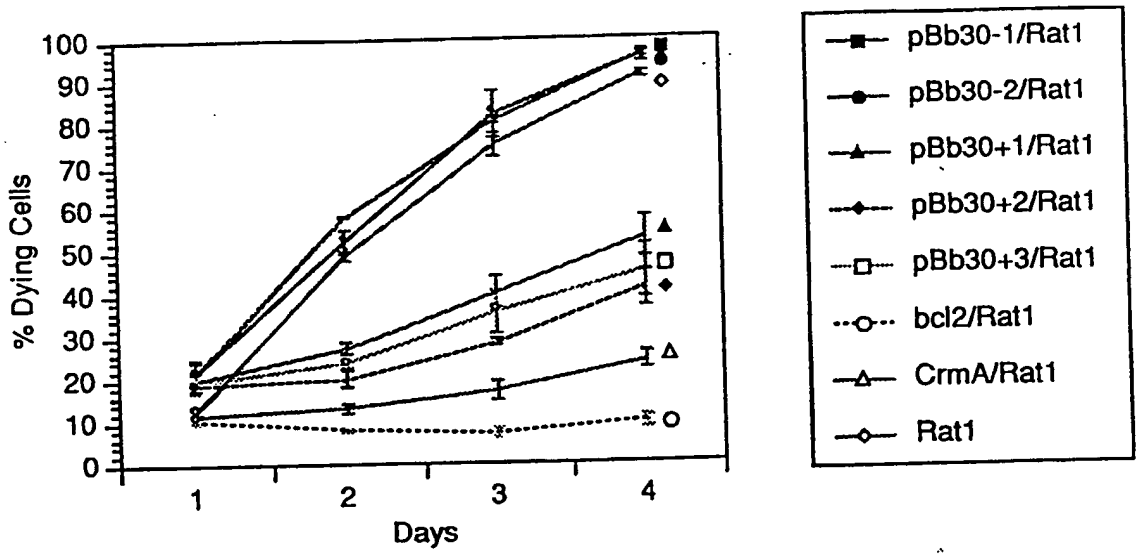


Figure 13

```
ICH-1S  ..............MHPHHQETLKKNRVVLAKQLLLSELLEHLLEKDIITLEMRELIQ.AKVGSFSQNVELLNLL 60
ICH-1L  MAADRGRRILGVCGMHPHHQETLKKNRVVLAKQLLLSELLEHLLEKDIITLEMRELIQ.AKVGSFSQNVELLNLL 74
hICE    MAD.........KVLKEKRKLFIRS.....MGEGTINGLLDELLQTRVLNKEEMEKVKRENATVMDKTRALIDSV 61
mICE    MAD.........KILRAKRKQFINS.....VSIGTINGLLDELLEKRVLNQEEMDKIKLANITAMDKARNLCDHV 61
Ced-3   ..............MMRQDRRSLLERNIMMFSSHLKVDEILEVLIAKQVLNSDNGDMIN.SCGTVREKRREIVKAV 61

ICH-1S  PKRGPQAFDAFCEALRETKQGHLEDML.....................................................L 88
ICH-1L  PKRGPQAFDAFCEALRETKQGHLEDML.....................................................L 102
hICE   IPKGAQACQ.ICITYICEEDSYLAGTLGLSADQTSGNYLNMQ.....................................DSQGVL 108
mICE   SKKGAPASQ.IFITYICNEDCYLAGILELQSAPSAETFVATE.....................................DSKGGH 108
Ced-3   QRPGDVAFDAFYDALRSTGHEGLAEVLEPLARSVDSNAVEFECPMSPASHRRSRALSPAGYTSPTRVHRDSVSSV 136

ICH-1S  TTLSGLQHVL.....................................PPLSCDYDLSLPFPVCESCPLYKKLRLSTDTVEHS 133
ICH-1L  TTLSGLQHVL.....................................PPLSCDYDLSLPFPVCESCPLYKKLRLSTDTVEHS 147
hICE    SSFPAPQAVQ.....................................DNPAMPTSSGSEGNVKLC.......SLEEA....QRI 144
mICE    PSSSETKE.E.....................................QNKEDGTFPGLTGTLKFC.......PLEKA....QKL 143
Ced-3   SSFTSYQDIYSRARSRSRSRALHSSDRHNYSSPPVNAFPSQPSSANSSFTGCSSLGYSSSRNRSFSKASGPTQYI 211
                                                                                F

ICH-1S  LDNKDGPVCLQVKPCTPEFYQTHFQLAYRLQSRPRGLALVLSNVHFTGEKELEFRSGGDVDHSTLVTLFKLLGYD 208
ICH-1L  LDNKDGPVCLQVKPCTPEFYQTHFQLAYRLQSRPRGLALVLSNVHFTGEKELEFRSGGDVDHSTLVTLFKLLGYD 222
hICE    WKQ......KSAEIYPIMDKSSR....TR.......LALIICNEEF...DSIPRRTGAEVDITGMTMLLQNLGYS 199
mICE    WKE......NPSEIYPIMNTTTR....TR.......LALIICNTEF...QHLSPRVGAQVDLREMKLLLEDLGYT 198
Ced-3   FHEEDMNFVDAPTISRVFDEKTM....YRNFSSPRGMCLIINNEHF...EQMPTRNGTKADKDNLTNLFRCMGYT 279

Nedd-2                                                  MLTVQV.YRTSQK.....CSSSKHVVEVLLDPLGTSFC.SL 34
ICH-1S  VHVLCDQTAQEMQEKLQNFAQLPAHRVTDSCIVALLSHGVEGAIYG......VDGKLLQLQEVFQLFDNANCPSL 277
ICH-1L  VHVLCDQTAQEMQEKLQNFAQLPAHRVTDSCIVALLSHGVEGAIYG......VDGKLLQLQEVFQLFDNANCPSL 291
hICE    VDVKKNLTASDMTTELEAFAHRPEHRTSDSTFLVFMSHGIREGICGKKHSEQVPDI.LQLNAIFNMLNTKNCPSL 273
mICE    VKVKENLTALEMVKEVKEFAACPEHRTSDSTFLVFMSHGIQEGICGTTYSNEVSDI.LKVDTIFQMMNTLKCPSL 272
Ced-3   VICKDNLTGRGMLLTIRDFAKHESH..GDSAILVILSHGEENVIIG......VDDIPISTHEIYDLLNAANAPRL 346
                    S                                                                T

Nedd-2  LPPPLLLY.......ETDRGVDQQDGKMHTQSP.........GCEESDAG.....KEELMKMRLPTRSDMICGYAC 89
ICH-1S  QNKPKMFFIQACRGGAIGSLGHLLLFTAATASL.........AL*                                312
ICH-1L  QNKPKMFFIQACRGDETDRGVDQQDGKNHAGSP.........GCEESDAG.....KEKLPKMRLPTRSDMICGYAC 353
hICE    KDKPKVIIIQACRGDSPGVVW.FKDSV..............GVSGNLSLPTTEEFEDDAI.KKAHIEKDFIAFCSS 333
mICE    KDKPKVIIIQACRGEKQGVVL.LKDSV..............RDSEE.DFLTDAIFEDDGI.KKAHIEKDFIAFCSS 331
Ced-3   ANKPKIVFVQACRGERRDNGFPVLDSVDGVPAFLRRGWDNRDGPLFNFLGCVRPQVQQVWRKKPSQADILIRYAT 421

Nedd-2  LKGNAAMRNTKRGSWYIKALTQVFSERACDMHVADMLVKVNALIK.EREGYAPGTEFHRCKEMSEYCSTLCQQLY 163
ICH-1L  LKGTAAMRNTKRGSWYIKALAQVFSKRACDMHVADMLVKVNALIK.DREGYAPGTEFHRCKEMSEYCSTLCRHLY 427
hICE    TPDNVSWRHPTMGSVFIGRLIEHMQEYACSCDVEEIF....RKVRFSFEQPDGRAQMPTTERVT.....LTRCFY 399
mICE    TPDNVSWRHPVRGSLFIESLIKHMKEYAWSCDLEDIF....RKVRFSFEQPEFRLQMPTADRVT.....LTKRFY 397
Ced-3   TAQYVSWRNSARGSWFIQAVCEVFSTHAKDMDVVELLTEVNKKVACGFQTSQGSNILKQMPEMTSR...LLKKFY 493

Nedd-2  LFPGYPPT*  171
ICH-1L  LFPGHPPT*  435
hICE    LFPGH*     404
mICE    LFPGH*     402
Ced-3   FWPEARNSAV* 503
```

Figure 14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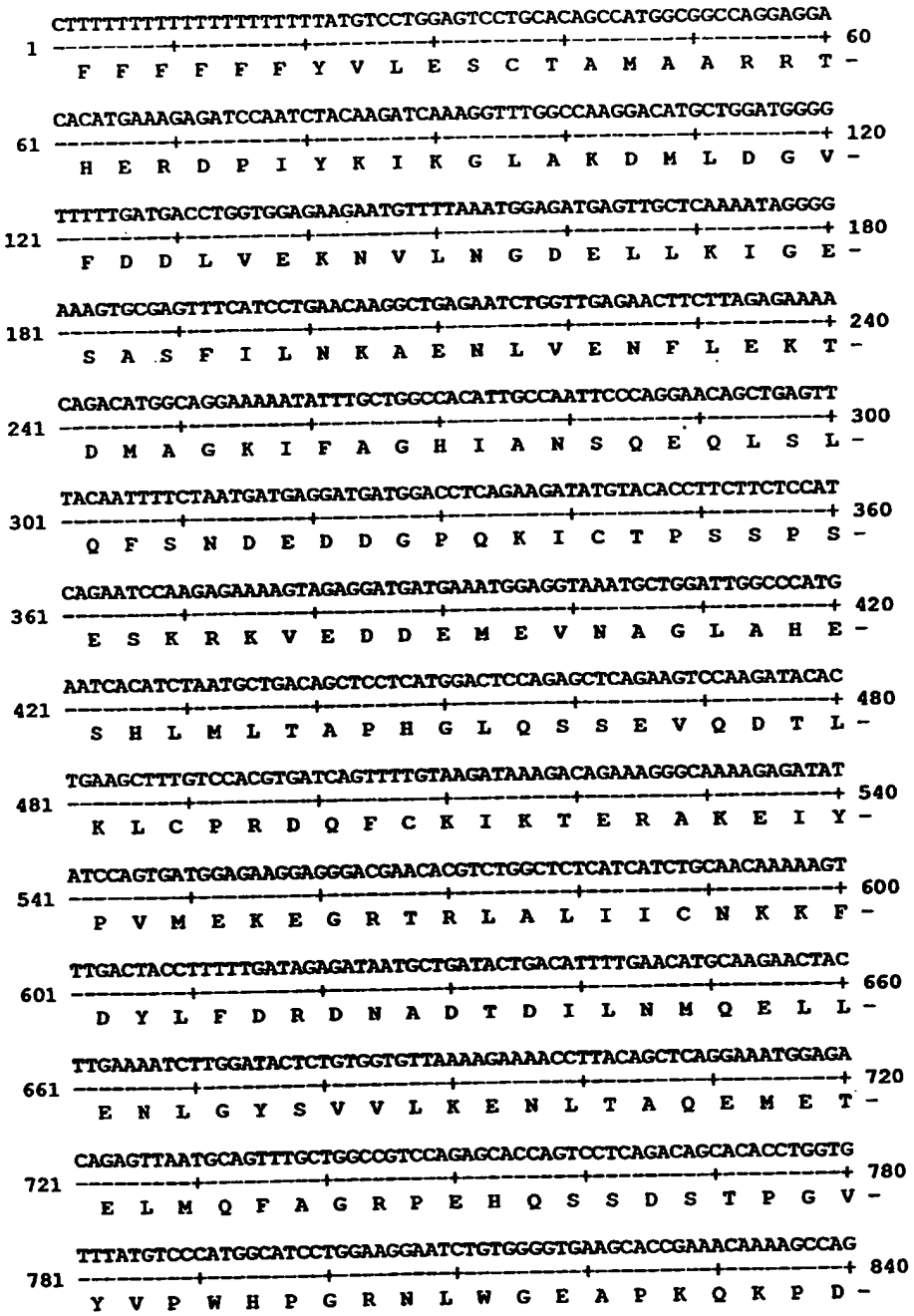
bcl2/Rat1
CrmA/Rat1
Rat1

Figure 15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61
120
H E R D P I Y K I K G L A K D M L D G V
TTTTTGATGACCTGGTGGAGAAGAATGTTTTAAATGGAGATGAGTTGCTCAAAATAGGGG
121
180
F D D L V E K N V L N G D E L L K I G E
AAAGTGCGAGTTTCATCCTGAACAAGGCTGAGAATCTGGTTGAGAACTTCTTAGAGAAAA
181
240
S A S F I L N K A E N L V E N F L E K T
CAGACATGGCAGGAAAAATATTTGCTGGCCACATTGCCAATTCCCAGGAACAGCTGAGTT
241
300
D M A G K I F A G H I A N S Q E Q L S L
TACAATTTTCTAATGATGAGGATGATGGACCTCAGAAGATATGTACACCTTCTTCTCCAT
301
360
Q F S N D E D D G P Q K I C T P S S P S
CAGAATCCAAGAGAAAAGTAGAGGATGATGAAATGGAGGTAAATGCTGGATTGGCCCATG
361
420
E S K R K V E D D E M E V N A G L A H E
AATCACATCTAATGCTGACAGCTCCTCATGGACTCCAGAGCTCAGAAGTCCAAGATACAC
421
480
S H L M L T A P H G L Q S S E V Q D T L
TGAAGCTTTGTCCACGTGATCAGTTTTGTAAGATAAAGACAGAAAGGGCAAAAGAGATAT
481
540
K L C P R D Q F C K I K T E R A K E I Y
ATCCAGTGATGGAGAAGGAGGGACGAACACGTCTGGCTCTCATCATCTGCAACAAAAAGT
541
600
P V M E K E G R T R L A L I I C N K K F
TTGACTACCTTTTTGATAGAGATAATGCTGATACTGACATTTTGAACATGCAAGAACTAC
601
660
D Y L F D R D N A D T D I L N M Q E L L
TTGAAAATCTTGGATACTCTGTGGTGTTAAAAGAAAACCTTACAGCTCAGGAAATGGAGA
661
720
E N L G Y S V V L K E N L T A Q E M E T
CAGAGTTAATGCAGTTTGCTGGCCGTCCAGAGCACCAGTCCTCAGACAGCACACCTGGTG
721
780
E L M Q F A G R P E H Q S S D S T P G V
TTTATGTCCCATGGCATCCTGGAAGGAATCTGTGGGGTGAAGCACCGAAACAAAAGCCAG
781
840
Y V P W H P G R N L W G E A P K Q K P D

Figure 16

```
    ATGTTCTTCATGATGACACTATCTTCAAAATTTTCAACAACTCTAACTGTCGGAGTCTGA
841 ---------+---------+---------+---------+---------+---------+ 900
     V  L  H  D  D  T  I  F  K  I  F  N  N  S  N  C  R  S  L  R -

    GAAACAAACCCAAGATTCTCATCATGCAGGCCTGCAGAGGCAGATATAATGGAACTATTT
901 ---------+---------+---------+---------+---------+---------+ 960
     N  K  P  K  I  L  I  M  Q  A  C  R  G  R  Y  N  G  T  I  W -

    GGGTATCCACAAACAAAGGGATAGCCACTGCTGATACAGATGAGGAACGTGTGTTGAGCT
961 ---------+---------+---------+---------+---------+---------+ 1020
     V  S  T  N  K  G  I  A  T  A  D  T  D  E  E  R  V  L  S  C -

    GTAAATGGAATAATAGTATAACAAAGGCCCATGTGGAGACAGATTTCATTGCTTTCAAAT
1021 ---------+---------+---------+---------+---------+---------+ 1080
     K  W  N  N  S  I  T  K  A  H  V  E  T  D  F  I  A  F  K  S -

    CTTCTACCCCACATAATATTTCTTGGAAGGTAGGCAAGACTGGTTCCCTCTTCATTTCCA
1081 ---------+---------+---------+---------+---------+---------+ 1140
     S  T  P  H  N  I  S  W  K  V  G  K  T  G  S  L  F  I  S  K -

    AACTCATTGACTGCTTCAAAAAGTACTGTTGGTGTTATCATTTGGAGGAAATTTTTCGAA
1141 ---------+---------+---------+---------+---------+---------+ 1200
     L  I  D  C  F  K  K  Y  C  W  C  Y  H  L  E  E  I  F  R  K -

    AGGTTCAACACTCATTTGAGGTCCCAGGTGAACTGACCCAGATGCCCACTATTGAGAGAG
1201 ---------+---------+---------+---------+---------+---------+ 1260
     V  Q  H  S  F  E  V  P  G  E  L  T  Q  M  P  T  I  E  R  V -

    TATCCATGACACGCTATTTCTACCTTTTTCCCGGGAATTAGCACAGGCAACTCTCATGCA
1261 ---------+---------+---------+---------+---------+---------+ 1320
     S  M  T  R  Y  F  Y  L  F  P  G  N  *
    GTTCACAGTCAAGTATTGCTGTAGCTGAGAAGAAAAGAAAATTCCAAGATCCCAGGATTT
1321 ---------+---------+---------+---------+---------+---------+ 1380
    TTAAATGTGTAAAACTTTT
1381 ---------+--------- 1399
```

Figure 16 (cont'd)

```
      1                                                            50
Ice4  .......... .........M AARRTHERDP IYKIKGLAKD MLDGVFDDLV
 Ice  .......... .......... MADKILRAKR KQFINSVSIG TINGLLDELL
Ice2  .......... .......... MAENKHPDKP LKVLEQLGKE VLTEYLEKLV
Ice3  IPHKELMAAD RGRRILGVCG MHPHHQETLK KNRVVLAKQL LLSELLEHLL
Ced3  .......... .........M MRQDRRSLLE RNIMMFSSHL KVDEILEVLI

      51                                                          100
Ice4  EKNVLNGDEL LKIGESASFI LNKAENLVEN FLEKTDMAGK IFAGHI.ANS
 Ice  EKRVLNQEEM DKIKLANITA MDKARNLCDH VSKKGAPASQ IFITYI.CNE
Ice2  QSNVLKLKEE DKQKFNNAER SDKRWVFVDA MKKKHSKVGE MLL.......
Ice3  EKDIITLEMR ELIQ.AKVGS FSQNVELLNL LPKRGPQAFD AFCEALRETK
Ced3  AKQVLNSDNG DMIN.SCGTV REKRREIVKA VQRPGDVAFD AFYDALRSTG

      101                                                         150
Ice4  QEQLSLQF.. .......... .......... .......... ..........
 Ice  DCYLAGIL.. .......... .......... .......... ..........
Ice2  .......... .......... .......... .......... ..........
Ice3  QGHLEDML.. .......... .......... .......... ..........
Ced3  HEGLAEVLEP LARSVDSNAV EFECPMSPAS HRRSRALSPA GYTSPTRVHR

      151                                                         200
Ice4  ....SNDEDD GPQKICTPSS PS........ .......... ....ESKRKV
 Ice  ....ELQSAP SAETFVATED SK........ .......... ....GGHPSS
Ice2  .......... ..QTFFSVD. .P........ .......... ....GSHHGE
Ice3  .....LTTLS GLQHV..... .......... .......... ...LPPLSCD
Ced3  DSVSSVSSFT SYQDIYSRAR SRSRSRALHS SDRHNYSSPP VNAFPSQPSS

      201                                                         250
Ice4  EDDEMEVNAG LAHESHL... MLTAPHGLQS SEVQDTLKLC PRDQFCKIKT
 Ice  SETKEEQNKE DGTFPGL... T.......... ....GTLKFC PLEKAQKLWK
Ice2  ANLEMEEPEE S.......... L.......... ....NTLKLC SPEEFTRLCR
Ice3  YDLSLPFPVC ESCPLYKKLR LSTDTVEHSL DNKDGPVCLQ VKPCTPEFYQ
Ced3  ANSSFTGCSS LGYSSSRNRS FSKASGPTQY IFHEEDMNFV DAPTISRVFD

      251                                                         300
Ice4  ERAKEIYPVM EKEGRTRLAL IICNKKF... DYLFDRDNAD TDILNMQELL
 Ice  ENPSEIYPIM NTTTRTRLAL IICNTEF... QHLSPRVGAQ VDLREMKLLL
Ice2  EKTQEIYPIK EANGRTRKAL IICNTEF... KHLSLRYGAK FDIIGMKGLL
Ice3  THFQLAYRLQ SR..PRGLAL VLSNVHFTGE KELEFRSGGD VDHSTLVTLF
Ced3  E..KTMYRNF SS..PRGMCL IINNEHF... EQMPTRNGTK ADKDNLTNLF

      301                                                         350
Ice4  ENLGYSVVLK ENLTAQEMET ELMQFAGRPE HQSSDSTPGV YVPWHPGRNL
 Ice  EDLGYTVKVK ENLTALEMVK EVKEFAACPE HKTSDSTFLV FMSHGIQEGI
Ice2  EDLGYDVVVK EELTAEGMES EMKDFAALSE HQTSDSTFLV LMSHGTLHGI
Ice3  KLLGYDVHVL CDQTAQEMQE KLQNFAQLPA HRVTDSCIVA LLSHGVEGAI
Ced3  RCMGYTVICK DNLTGRGMLL TIRDFAKHES H..GDSAILV ILSHGEENVI

      351                                                         400
Ice4  WGEAPKQK.P DVLHDDTIFK IFNNSNCRSL RNKPKILIMQ ACRGRYNGTI
 Ice  CGTTYSNEVS DILKVDTIFQ MMNTLKCPSL KDKPKVIIIQ ACRGEKQGVV
Ice2  CGTMHSEKTP DVLQYDTIYQ IFNNCHCPGL RDKPKVIIVQ ACRGGNSGEM
Ice3  YGVD.....G KLLQLQEVFQ LFDNANCPSL QNKPKMFFIQ ACRGDETDRG
Ced3  IGVD.....D IPISTHEIYD LLNAANAPRL ANKPKIVFVQ ACRGERRDNG
```

Figure 17

```
      401                                                        450
Ice4  WVSTNKGIAT A....DTDEE RVLSCKWNNS ITKAHVET.. .......DFI
 Ice  LLKDSVRD.. .....SEEDF LTDAIFEDDG IKKAHIEK.. .......DFI
Ice2  WIRESSKPQL C....RGVDL PRN..MEADA VKLSHVEK.. .......DFI
Ice3  VDQQD..... ......GKNH AGSPGCEESD AGKEKLPKMR ..LPTRSDMI
Ced3  FPVLDSVDGV PAFLRRGWDN RDGPLFNFLG CVRPQVQQVW RKKPSQADIL

      451                                                        500
Ice4  AFKSSTPHNI SWKVGKTGSL FISKLIDCFK KYCWCYHLEE IFRKVQHSFE
 Ice  AFCSSTPDNV SWRHPVRGSL FIESLIKHMK EYAWSCDLED IFRKVRFSFE
Ice2  AFYATTPHHL SYRDKTGGSY FITRLISCFR KHACSCHLFD IFLKVQQSFE
Ice3  CGYACLKGTA AMRNTKRGSW YIEALAQVFS ERACDMHVAD MLVKVNALIK
Ced3  IRYATTAQYV SWRNSARGSW FIQAVCEVFS THAKDMDVVE LLTEVNK..K

      501                                              540
Ice4  VPGELTQMPT IERV..... ..SMTRYFYL FPGN*.....
 Ice  QPEFRLQMPT ADRV..... ..TLTKRFYL FPGH.....
Ice2  KASIHSQMPT IDRA..... ..TLTRYFYL FPGN*.....
Ice3  DREGYAPGTE FHRCKEMSEY CSTLCRHLYL FPGHPPT...
Ced3  VACGFQTSQG SNILKQMPEM TSRLLKKFYF WPEARNSAV*
```

Figure 17 (cont'd)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06630

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/02, 37/54, 48/00; C07K 15/06; C12N 15/57
US CL :514/12, 21, 44; 435/226, 320.1; 536/23.2, 24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 21, 44; 435/226, 320.1; 536/23.2, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, SWISSPROT, PIR, EMBL, GENBANK, search terms; NEDD2, ICH1, ICE4, ICE, MICE2, PROGRAMMED CELL DEATH, CONVERTING ENZYME, crmA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Cell, Volume 69, issued 15 May 1992, Ray et al., "Viral inhibition of inflammation: cowpox virus encodes an inhibitor of the interleukin-1beta converting enzyme", pages 597-604, see the whole publication, especially the abstract.	1-3
X	Science, Volume 256, issued 03 April 1992, Cerretti et al., "Molecular cloning of the interleukin-1beta converting enzyme", pages 97-100, see whole publication, especially page 98.	14, 20, 25
X	UEMBL, Accession number D28492 and D10713, issued 04 June 1994, Kumar et al., "Mouse mRNA for Nedd2 protein", see whole abstract, especially the sequence.	20

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 03 OCTOBER 1994	Date of mailing of the international search report 06 OCT 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KEITH FURMAN, PH.D. Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06630

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	Bio/Technology, Volume 11, issued July 1993, Edgington, "Looking death in the eye: apoptosis and cancer research", pages 787-792, see column 1 of page 788 and column 2 of page 790.	1-25
A	Chemical Reviews, Volume 90, Number 4, issued June 1990, Uhlmann et al., "Antisense oligonucleotides: a new therapeutic principle", pages 543-584, see whole publication, especially column 2 of page 561.	1-25